

PB-218 284

PROCEDURES FOR THE ISOLATION AND IDENTIFICATION OF MYCOBACTERIA

Annie L. Vestal

National Communicable Disease Center
Atlanta, Georgia

June 1969

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ERRATA NOTICE
PROCEDURES FOR THE
ISOLATION AND IDENTIFICATION OF MYCOBACTERIA
PHS PUBLICATION #1995

- Page 11 Under "Tuesday"
Now Reads: Read MacConkey, 5 days old
Should Read: Read MacConkey, 11 days old
- Page 53 Now Reads: 0.1% aspartic acid
Should Read: 0.1% potassium aspartate
- Page 57 Now Reads: "f. Results"
Should Read: d. Results
- Page 66 Now Reads: appendix D page 137
Should Read: appendix D page 109
- Page 79 Now Reads: Section C, Reporting Drug Susceptibility Tests.
Should Read: Section D, Reporting Drug Susceptibility Tests.
- Page 83 Under Test b
Now Reads: INH 0.02 mcg/ml
Should Read: INH 0.2 mcg/ml
- Page 85 Sample Laboratory Report Form
Now Reads: Growth at Dils. 10^{-2} , 10^{-4}
Should Read: Growth at Dils. 10^{-1} , 10^{-3}
- Page 93 Now Reads: Dissolve 100 mg. of rifampin in 10.0 ml distilled water.
Should Read: Dissolve 100 mg of rifampin in 2 ml of ethanol or Di Methyl Sulfoxide, then make up to 10.0 ml volume with distilled water.

Now Reads: For storage of drugs, see Table I
Should Read: For storage of drugs, see Table III.
- Page 118 Now Reads: preparing Lowenstein medium, page
Should Read: preparing Lowenstein medium, page 117.

**PROCEDURES
FOR THE ISOLATION
AND IDENTIFICATION
OF MYCOBACTERIA**

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U. S. DEPARTMENT OF HEALTH, EDUCATION,
AND WELFARE
Public Health Service
HEALTH SERVICES AND MENTAL HEALTH ADMINISTRATION
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Public Health Service Publication No. 1995
June, 1969.

UNITED STATES GOVERNMENT PRINTING OFFICE
WASHINGTON, D. C.: 1969

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Introduction

This manual has been prepared for the laboratorian who is charged with the technical responsibility of isolating and identifying the mycobacteria commonly encountered in clinical specimens. It contains detailed instructions for each procedure. The overall philosophy in the laboratory aspects of the mycobacterioses as well as a more complete bibliography are found in:

Kubica, G. P. and Dye, W. E. 1967. "Laboratory Methods for Clinical and Public Health Mycobacteriology", Public Health Service Publication No. 1547, U. S. Government Printing Office, Washington, D. C. 20402.

In a work of this nature it is impossible to acknowledge all of those who have, in one way or another, contributed the needed material, advice, and stimulation. However, to Dr. George P. Kubica and other members of the staff in the Tuberculosis Unit of NCDC, goes my personal appreciation for their review, criticisms, and other contributions. To Dr. U. Pentti Kokko, Director, Laboratory Division, and Dr. Phyllis Q. Edwards, Chief, Tuberculosis Branch, NCDC, I express my sincere thanks for their encouragement and support in making this manual possible.

SECTION I. SAFETY IN THE LABORATORY

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A. INTRODUCTION TO SECTION I

Each laboratory worker must be responsible for his own safety and that of his co-workers.

To avoid accidental infection:

Do not take unnecessary risks.

Use safety equipment properly.

Be prepared to act quickly in case of accident.

Pulmonary diseases such as tuberculosis are known to be spread by airborne transmission. Many manipulations in the laboratory create aerosols that may lead to human infection. Some common sources of aerosols are:

- Opening specimen containers
- Pipetting
- Flaming loops
- Centrifuges
- Shaking machines
- Blenders
- Syringes
- Animal autopsy
- Opening lyophilized ampoules

Specially designed suites for tuberculosis bacteriology are of no value unless they are properly used. Proper control depends more on human factors than on laboratory design or the development of new procedures and equipment. Administrators should see that the essential equipment is available and properly used.

Safety measures described here are an accumulation of common sense practices devised by experienced personnel. With careful planning, an efficient routine may be set up without sacrificing safety, accuracy, or speed.

B. PERSONNEL

Personnel should be selected with care. They should be physically and mentally capable. Fatigue, emotional stress, and malnutrition contribute to the development of tuberculosis following exposure.

Before working in the laboratory, all personnel should have (1) a tuberculin skin test, (2) a chest X-ray, and (3) training in safety techniques and procedures.

While employed in the tuberculosis laboratory tuberculin negative personnel should be retested at 3- to 6-month intervals, and all employees should have chest X-rays at 3- to 6-month intervals.

If an employee converts from skin test negative to positive, the following should be done:

The employee should be referred to a physician for possible isoniazid chemoprophylaxis.

The employee should check his own personal habits.

The laboratory should re-evaluate its safety equipment and procedures.

Remember, however, that even the best safety equipment is no substitute for meticulous technique.

C. EQUIPMENT

Alcohol-sand flask (Figure 1)

Biological safety cabinet (hood)

Burner, Bunsen or Fisher type

Centrifuge safety cups, aerosol free

Discard pans with covers

Disinfectant for hands, 2% amphyll or other

Disinfectant, liquid phenol.

Fogging machine for room decontamination*

Hats, paper, disposable

Hospital gowns

Masks, disposable

Rubber gloves, disposable

Safety pipetting devices

Shoe covers, plastic, or autoclavable sneakers

Splash-proof containers for fluids (Figure 2)

Tissue grinder, Ten Broeck, hand

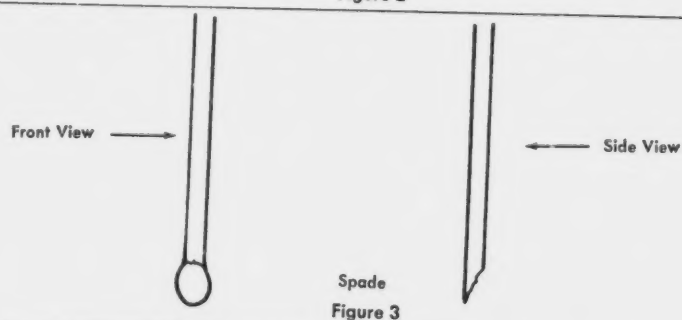
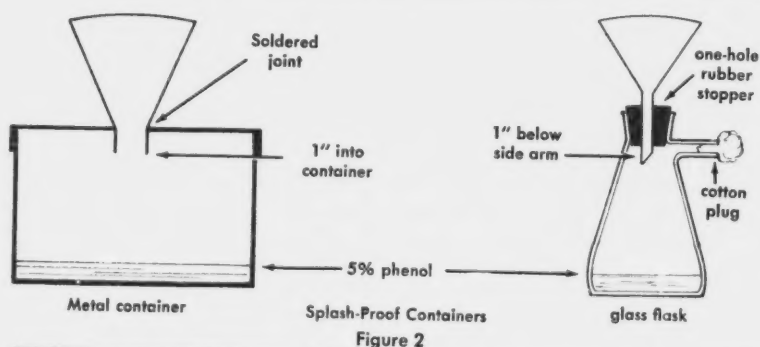
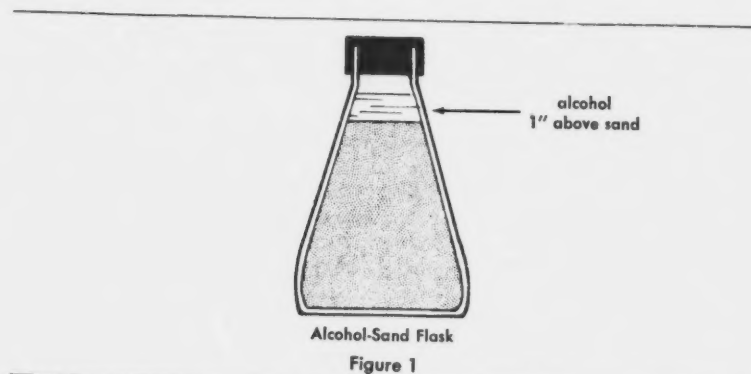
Ultrafilters for hood

Ultraviolet lamps in hoods

Ultraviolet single sheet paper sterilizer (or autoclave specimen slips in a paper bag)

Wire, 18-gauge nichrome for inoculation "spade" (Figure 3)

* Oxford Hospital Fogging Unit and Turntable from Center Chemical Co., 5001 Peachtree Industrial Blvd., Chamblee, Georgia.



A spade may be made by flattening the end of a nichrome wire (18 gauge). The flattened area should be 5 to 10 mm long by 0.5 mm thick.

A heavyweight wire is normally used for making inoculating loops and spades for use with the alcohol-sand flask. Lightweight wire has a tendency to bend on contact with the sand.

D. GUIDELINES

1. A Layout For A Mycobacteriology Laboratory

DEFINITIONS:

Contaminated Area(s): Any room(s) in which specimen containers are opened, cultures are prepared or transferred, and animals are inoculated, housed after inoculation, or autopsied.

Contaminated Equipment: *All* equipment used within a contaminated area, e.g., specimen containers, needles, syringes, pipettes, metal trays, discard pans, culture tubes, centrifuges, autopsy equipment, pencils, pens, and culture request slips.

GENERAL REQUIREMENTS:

A completely self-contained suite housing all equipment needed for processing specimens from receipt to final report. (See Figure 4.)

A ventilation system engineered to maintain airflow in one direction only, i.e., from a clean area into a less clean area. Keep a continuous check on airflow by means of short paper strips in areas of rapid flow (air duct openings or grills in doors and walls).

An exhaust system (with bacteriologic filters) discharging air to the outside of the building at the roof or side into an area where the possibility of return to the building is minimal.

A separate room, within the suite (see Figure 5), designated as the Isolation Room or Contamination Area and containing a biological safety cabinet for the opening of specimens, digestion of sputa, preparation of slides, and inoculation of cultures.

2. Basic Requirements For An Efficient Biological Safety Cabinet

Exhaust fan:

With the capacity to draw a minimum of 50 to 75 lineal feet of air per minute across the entire front opening.

Operate during all manipulations with specimens, animals, and cultures.

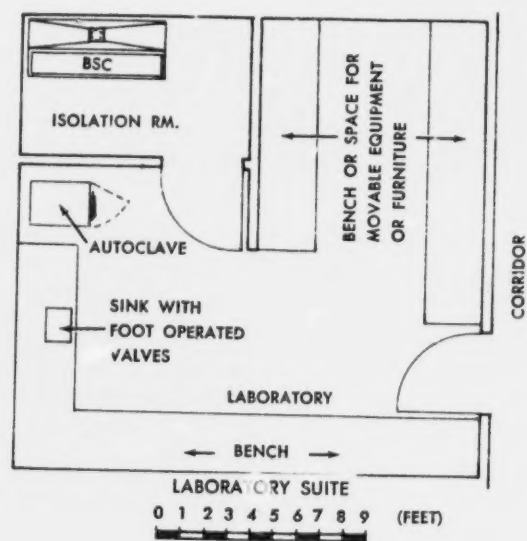


Figure 4

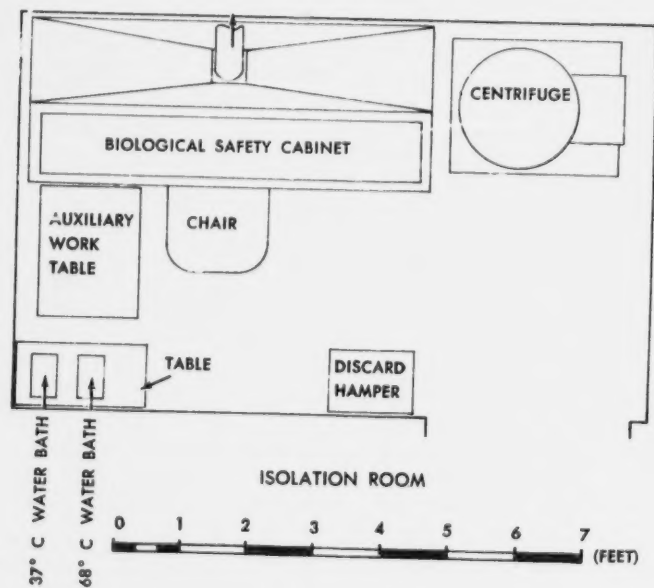


Figure 5

Check airflow at least every 3 months; airflow rate gauges may be mounted on the cabinet to continuously monitor the airflow.

Ultraviolet lights:

These should be mounted within the working area of the cabinet.

Do not operate while the cabinet is in use, since direct or reflected UV rays are injurious to the skin, eyes, and also to the mycobacteria.

Turn on immediately after finishing the work in the cabinet and allow to remain on for a minimum of 2 hours. Cost of continuous operation when the cabinet is not in use is minimal and may be more convenient.

Warning: *Ultraviolet energy has very little penetrating power. These lights serve only as a supplement to chemical disinfection and careful techniques.*

Cleanse at least every 2 weeks with ethyl alcohol to remove residues and dust.

Check at least every 3 months for efficiency of ultraviolet output. (UV lamps may be checked with a Westinghouse Model SM-600 Meter or the equivalent. Visible light is *not* a reliable indicator of ultraviolet output.)

No safety cabinet is 100% effective.

There is no substitute for care in performing all techniques.

3. General Safety Requirements

- a. Prohibit smoking and eating in the tuberculosis laboratory.
- b. Do NOT handle or touch any contaminated materials with bare hands. If this inadvertently occurs, rinse hands in a disinfectant solution and then wash them well with soap and water or 2% amphyl.
- c. Always carry cultures in a rack or basket. Tubes and plates easily slip out of the hands, especially when wet.
- d. Cover any broken cultures with cloth or paper towels and flood with 5% phenol. To prevent dried particles from becoming airborne, keep the area wet with phenol and allow the material to stand at least an hour before handling.
- e. Keep a container of 5% phenol in every area where cultures are handled or stored. **BE PREPARED.**

- f. Autoclave all contaminated materials before sending them to the washroom. Loosen the screw caps of culture tubes to allow steam to penetrate.
- g. Use sterility controls (Diack Sterilizer Control or an equivalent) to insure proper functioning of autoclaves.
- h. Maintain regular checks on the biological safety cabinet's exhaust system and UV light efficiency.
- i. Permit no servicing, cleaning, or checking of isolation room equipment unless a trained technical or professional person is present to insure that adequate safety precautions are observed.
Before major servicing, such as changing filters, decontaminate the entire room by fogging, then wash down the ceiling, walls, floor, and all equipment with 5% aqueous solution of phenol or an equally effective disinfectant.
- j. Let no one enter the Contaminated Area in the event of gross contamination (as by breakage of culture tubes) until the entire area has been decontaminated by individuals within the area, unless an injured person requires emergency care.

4. Safety Precautions Before Entering The Isolation Room

- a. Assemble all materials and equipment. Label specimens, tubes for processing, media for inoculation, etc., and place them on a cart or table just outside the isolation room door.
- b. Have extra supplies, such as media, saline, sterile pipettes, and reagents, within easy reach from the isolation room.
- c. Plan other work to do while in the laboratory or while waiting for the centrifuge, e.g., culture-transfers and smears.
- d. Be organized. Keep a check list, if necessary.
- e. Put on protective clothing such as a cap, gown, an effective mask, leak-proof gloves, disposable or autoclavable shoe covers or sneakers.

ONCE IN THE ISOLATION ROOM, DO NOT LEAVE UNTIL YOUR TASK IS COMPLETED. If you need additional equipment, ask someone to get it for you.

5. Safety Precautions Within The Isolation Room

- a. Consider everything within the limits of the Isolation Room as potentially infectious. Let *no one* enter without full protective clothing. Organisms on contaminated surfaces may enter the body through cuts and abrasions in the skin.
- b. Immediately after *each* use, place all protective clothing in a covered discard hamper. Autoclave hamper before sending clothing to laundry and before discarding disposable items.
- c. Perform *all* work over a gauze pad or towel soaked with 5% phenol solution. When work is completed, place the gauze pad in a covered pan containing 5% phenol solution.
- d. Before opening a specimen container, disinfect the outside by wiping it with gauze soaked in 5% phenol solution. (The container may have been contaminated when the specimen was collected or during transit.)
- e. Autoclave inner metal mailing tube before reuse because it may be contaminated.
- f. Place laboratory request slips accompanying specimens in a paper bag and autoclave before the slips are handled by the record clerk.
- g. Open all tubes and containers with infectious materials within safety cabinet.
- h. Any operations involving agitation of specimens or cultures should be performed in aerosol-free containers or within the safety cabinet.
- i. Use *only* aerosol-free safety carriers in centrifuge.
- j. Heat fix all smears on a slide warmer for at least 2 hours at 65°C. Overnight is acceptable. (Shield slides from UV light rays.)
- k. *Before* flaming, clean inoculating loops and spades in an alcohol-sand flask. (See Figures 1 and 3.)
- l. *Always* use safety pipetting devices.
DO NOT mouth pipette under any circumstances.
- m. Place *all* materials to be discarded in covered pans containing 5% phenol solution.
- n. Discard *all* contaminated fluids into a splash-proof container with a small amount of 5% phenol solution. Autoclave container before disposing of contents.
- o. Place autopsied animals in leak-proof plastic bags, close bags *securely*, and deposit them in a covered metal container for transport to *incinerator*.

- p. When work is completed and BEFORE leaving the room:
- (1) Place covers on all discard pans. Contaminated material must be in covered containers when brought out of the isolation room.
 - (2) Wipe the outsides of the discard containers and culture tubes with disinfectant-soaked gauze before placing them outside the isolation room.
 - (3) Swab around the work area with 2% creosol or 5% phenol. Scrub the area periodically with soap and water to remove disinfectant residue.
 - (4) Place the gauze pad from the work area in a covered container containing phenol. Do not allow pad to dry under the hood.
 - (5) Cover the slides on the warmer to protect them from UV light.
 - (6) Turn on the UV lights in the hood.
 - (7) Remove protective clothing (MASK LAST) and place in a covered container for autoclaving before laundering.

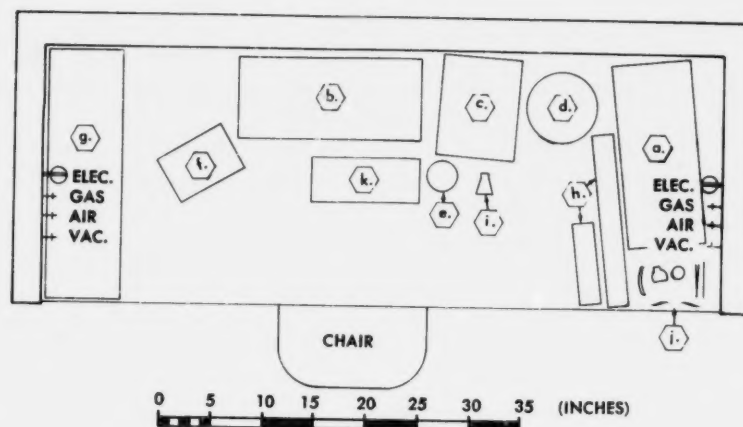
UPON LEAVING THE ROOM, *scrub hands and arms well with soap and water. Do not wear watches or bracelets while working in the hood because they interfere with thorough cleansing of hands and arms.*

6. Arrangement of Equipment In A Biological Safety Cabinet
(See Figure 6.) Convenient for right handed individuals.

NOTE: *This arrangement leaves a clear area immediately in front of the worker for handling specimens. Thus, any drops or invisible spatter are more likely to fall on the gauze, not the work.*

Cover work area surface with disinfectant-soaked gauze pad or several layers of disposable material. Make gauze pad 4-6 layers thick and about 18" x 36" wide. Sew around edge, keep in covered pan of disinfectant, squeeze out, and spread over work area. Gauze should be autoclaved at least once a week. It may be rinsed, dried, and reused.

Place supplies such as sterile saline and labeled media on shelves, table, or cart adjacent to the hood. Move to work area as needed.



BIOLOGICAL SAFETY CABINET WORK SURFACE

Figure 6

A key to letters on Figure 6 follows:

- a. Discard pan for reusable pipettes.
- b. Discard pan for test tubes, flasks, etc.
- c. Discard container for disposables, plastic, paper, etc.
- d. Splash-proof waste container for contaminated liquids (Figure 2).
- e. Bunsen burner. The rubber connecting hose should be long enough for the flame to be within easy reach. Avoid over-heating the room by keeping flame low.
- f. High-speed mixer to left of worker. Move to rear after processing specimens, if space is needed for work area.
- g. Slide warmer to extreme left.
- h. Pipette cannisters at right.
- i. Sand-flask with alcohol for cleaning spade or loop (Figures 1 and 3).
- j. Safety pipetting devices, interval timers, pencils, loops, forceps, and other small equipment.
- k. Racks for tubes during processing, etc.

**SECTION II. ISOLATION AND IDENTIFICATION OF
MYCOBACTERIA**

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II. A. DAILY WORK SCHEDULE

	IN REGULAR LABORATORY	IN BIOLOGICAL SAFETY CABINET
Each Day	Number clinical specimens. Label tubes, media, and slides. Prepare reagents. Stain and read slides. Send out reports. Incubate cultures.	Process clinical specimens. Make smears. Inoculate media. Do drug tests.
Monday	Read and record: Tween test, 5 and 12 days, Tellurite, Arylsulfatase.	(Process clinicals.) Add Na carbonate to arylsulfatase.
Tuesday	Read all cultures.* Record: growth rate, number of colonies, morphology, pigment. Set aside cultures for tests. Expose non-chromogenic to light for 1-hour and reincubate. Read MacConkey, 5 days old. Set-up tests to be done on Wednesday.	(Process clinicals.) (Process clinicals.)
Wednesday	Read MacConkey, 5 days old. Read NaCl tolerance. Record test results: niacin, nitrate, catalase. Read 1-hour light.	(Process clinicals.) From cultures Tuesday: Inoculate Tween test. Perform niacin, nitrate, catalase tests. Make other necessary culture transfers.
Thursday	Read cultures, if necessary. Read Tween test, 24 hours. Set-up tests to be done on Friday. Check reagent supply.	(Process clinicals.)
Friday	Read Tween test, 9 days. Check cultures inoculated during the week for rapid growers.	(Process clinicals.) Transfer to liquid medium: Rapid Growers Slow Growers for Tellurite Inoculate 7-day old liquid cultures to arylsulfatase, MacConkey, NaCl medium. Add tellurite to 7-day culture.

* When cultures must be read more than 1 day a week, perform in vitro tests on Monday and/or Wednesday.

II. B. SPECIMENS

B. SPECIMENS

Specimens must be collected in clean, sterile containers and sent directly to the laboratory for immediate processing. If possible, specimens should be refrigerated during transit to the laboratory. Refrigeration discourages the multiplication of rapidly growing non-acid-fast organisms that reproduce at room temperature and sometimes make decontamination of the specimen impossible in the laboratory.

The specimen container must be clearly labeled with the patient's name and/or hospital number and the date the specimen was taken.

1. Types

a. SPUTUM

These specimens should be a series of 3 to 5 single early morning samples. A volume of 5 to 10 ml is adequate for each sample. There is no advantage in collecting a larger volume. Any volume, no matter how small, should be processed. Keep a record of volumes less than 2 ml and enter this information on the report form. It may be of value to the physician.

b. NEBULIZED "SPUTUM"

These specimens are usually very watery and should be labeled as "nebulized" so that they will not be discarded as saliva.

c. GASTRIC LAVAGE

These specimens must be processed within 4 hours after collection. When transportation must be delayed, the specimen may be neutralized by adding buffer tablets or 10% sodium carbonate to the cup in which the specimen is collected. An indicator will assist in determining neutrality.

d. URINE

A series of single mid-stream specimens, voided in the early morning, are more satisfactory than the 24-hour pooled specimen.

e. TISSUE

Any tissue to be processed must be collected aseptically and transported to the laboratory at once. When a delay is necessary, freeze the tissue and transport to the laboratory in the frozen state.

f. OTHER TYPES

These include aseptically collected specimens, such as: blood, pleural fluid, bronchial washings, pus, joint fluid, and laryngeal

swab. When necessary, sterile anticoagulants, such as heparin or ammonium oxalate, may be used.

2. Transportation

Any specimen received in a container or carton not conforming to United States Postal regulations should be reported to the administrative office or the responsible individual.

Cultures should be shipped to reference laboratories on solid medium in screw-capped tubes. Petri dish cultures are not to be shipped.

Anyone who knowingly deposits for mailing or delivery any package which does not fulfill the requirements is subject to severe penalties of fine or imprisonment. The burden of assuring compliance with the law rests with the mailer.

U. S. Public Health Service Publication No. 976, *Collection, Handling, and Shipment of Microbiological Specimens*, (Revised, 1968) contains specific directions for packing and mailing mycobacterial specimens.

U. S. Postal requirements for shipping disease¹ tissue and other specimens found in the *United States Postal Manual*, Parts 124 and 125, dated November 19, 1965, and Part 221, dated August 30, 1968 are printed in Appendix A, pages 99-103.

II. C. ISOLATION PROCEDURES

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C. ISOLATION PROCEDURES

1. Digestion and Decontamination

Use an effective but gentle technique such as the N-acetyl-L-cysteine (NALC) to digest and decontaminate specimens. However, through research and evaluation studies a more effective method may be developed, and, if so it should be used.

Number the specimens and the patient record slip with the laboratory accession number. Place the record slips in a paper bag or a special container to be autoclaved before being handled by the record clerk.

Swab the outside of the specimen container with 5% phenol.

a. EQUIPMENT AND SUPPLIES

Sterile centrifuge tubes with water-tight screw caps, 50 ml.

Sterile pipettes: Capillary, 1 ml, 10 ml, or other.

Microscope slides.

Media: 1 bi-plate of Middlebrook 7H-10 agar (7H-10) and 2 to 4 tubes of Lowenstein-Jensen egg medium (L-J) per specimen.

b. SOLUTIONS

For preparation, see Appendix E, page 111.

- (1) Necessary amount of NALC digestant-decontaminant for the specimens to be processed. Prepare each day.

For each 100 ml, combine:

- (a) 50 ml sterile 4% NaOH.
- (b) 50 ml sterile 2.9% Na citrate.
- (c) 0.5 g N-acetyl-L-cysteine powder.

Use this solution within 24 hours. Any excess solution may be stored in a tightly stoppered container and refrigerated for use the following day.

- (2) Sterile distilled water or pH 6.8 buffer. Prepare at least 30 ml per specimen when processing in 50 ml tubes.
- (3) Sterile bovine albumin 0.2% at pH 6.8.
- (4) Sterile water or 0.85% saline in screw-cap tubes, 4.5 ml each.

c. PREPARATIONS

Before entering the isolation room, label all media, slides, and centrifuge tubes with specimen numbers. The date of inoculation should be placed on the culture tubes.

Assemble all specimens, solutions, and supplies and place these on a cart or table by the door to the isolation room.

Put on protective clothing and enter the isolation room. THEN transfer the work and supplies into the work area. DO NOT LEAVE the area until the work is finished.

Make any necessary break in the procedure *after* adding the bovine albumin to the sediment. Refrigerate the sediment.

Perform All Manipulations Under The Hood. Cover Bench Top Work Area With A Disinfectant-Soaked Gauze Pad.

d. SPUTUM

- (1) Line up specimens and centrifuge tubes in numerical order.
- (2) Loosen caps of specimen containers (work in "sets" equivalent to one centrifuge load).
- (3) Transfer sputum to centrifuge tubes. Do not put more than 10 ml of sputum in a 50 ml tube. The amount of the specimen processed in the centrifuge tube must never be more than $\frac{1}{5}$ the volume of the tube.

When it is necessary to process the entire specimen, use more than one tube and combine the sediments after centrifugation (Step 9).

- (4) Add to the specimen a volume of NALC digestant *equal to but not more than* the amount of the specimen.
If the volume is very small or the specimen is extremely mucoid, add the NALC digestant to the specimen in the container and then transfer to the centrifuge tube.
- (5) Tighten caps on centrifuge tubes. Beginning with the first specimen in the series, mix each specimen on the Vortex until liquefied. This may take from 5-20 seconds per specimen.
- (6) Set timer for 15 minutes as soon as 1 or 2 specimens have been liquefied.
- (7) Let each specimen stand for 15 minutes.

Work at such speed that each specimen stands for 15 minutes and not more than 20 minutes before adding buffer.

- (8) Fill each tube to within $\frac{1}{2}$ inch of the top with pH 6.8 buffer. Tighten cap and swirl by hand to mix.

- (9) Centrifuge at 3000 rpm (1800-2400 *g*) for 15 minutes.
- (10) Pour off all of the supernatant into a splash-proof can containing a disinfectant. Flame lips of tubes to prevent contamination, or wipe lip of tube with a cotton pledget soaked with 5% phenol. Use one pledget per specimen. Do not let any phenol run down the inside of the specimen tube.
- (11) With a sterile pipette, add 1 or 2 ml of 0.2% bovine albumin to each sediment. Shake gently by hand to mix. (CAUTION: Vigorous shaking creates aerosol.)
- NOTE: Adjusting the pH of the sediment is not necessary because of the well-buffered media. Do not over-inoculate, since this interferes with the buffer action and results in poor growth.*
- (12) Using a sterile capillary pipette, mix sediment and make a 1:10 dilution by adding 0.5 ml (10 drops) to 4.5 ml sterile water or saline. Mix with the pipette, and:
- (a) Place 0.1 ml (or 2 drops) of the 1:10 dilution onto the surface of one-half of a bi-plate of 7H-10 medium, and place 2 drops on the surface of each of 2 tubes of L-J medium.
Spread with the pipette.
 - (b) Place 2 drops of the undiluted albumin sediment on one-half of the bi-plate of 7H-10 and 2 drops on each of 2 tubes of L-J medium.
 - (c) With a bent glass rod, spread inoculum over the surface of the 7H-10*. (Spread the 1:10 dilution and then the undiluted.) Inocula on plates may be left without spreading. In such cases 3 drops per half plate are recommended.
 - (d) Make a smear of the undiluted albumin sediment. Spread one drop over an area of 1 x 2 cm on the microscope slide. Discard pipette. Place slide on an electric slide warmer at 65°-75° C for 2 hours (overnight is acceptable).
- (13) Refrigerate excess undiluted albumin-sediment for direct drug tests. (See page 80.)

* A disposable bent glass rod is quickly made by heating the capillary portion of a pipette in the gas flame to form a right angle.

To Sputum From Patients Whose Specimens are Consistently Contaminated with Pseudomonas

- (1) Add equal volume 5% oxalic acid.
- (2) Mix on test tube mixer. Let stand R° 30 minutes. Shake occasionally.
- (3) Add sterile saline to both lower the specific gravity and dilute the acid.
- (4) Centrifuge 3000 rpm (1800-2400 *g*) 15 minutes.
- (5) Decant. Retain sediment.
- (6) Add an indicator and neutralize with 4% NaOH.
- (7) Inoculate as described in (12) above.

e. LARYNGEAL SWAB

- (1) With sterile forceps, transfer the swab to a sterile centrifuge tube.
- (2) Add 2 ml sterile distilled water or saline.
- (3) Add 2 ml NALC digestant.
- (4) Swirl on Vortex mixer.
- (5) Let stand 15 minutes to decontaminate.
- (6) With sterile forceps, remove swab from centrifuge tube.
- (7) Fill the tube to within 1½ inch of top with sterile buffer.
- (8) Centrifuge and continue as for sputum.

f. GASTRIC LAVAGE

Mucoid gastric lavage:

- (1) To 20-50 ml of gastric lavage add 50-100 mg ("pinch") of NALC powder.
- (2) Mix by hand or with test tube mixer as for sputum.
- (3) Centrifuge at 3000 rpm for 30 minutes.
- (4) Decant supernatant fluid and re-suspend sediment in 2-5 ml of sterile distilled water.
- (5) Add an equal volume of NaOH -NALC digestant and process as for sputum.

Fluid gastric lavage:

- (1) Centrifuge entire specimen for 30 minutes at 3000 rpm.
- (2) Decant and re-suspend in 2-5 ml of sterile distilled water.
- (3) Pool sediments if more than one container is used for centrifugation.
- (4) Add an equal volume of NALC-NaOH digestant and process as for sputum.

g. TISSUE

- (1) Grind with sterile tissue grinder in sterile 0.85% saline or 0.2% bovine albumin. When tissue contains mucous (e.g., lung), add a pinch of N-acetyl-L-cysteine.

- (2) If collected aseptically, inoculate directly to both liquid and solid media.
- (3) Smear liquid culture once a week until acid-fast bacilli are seen. Inoculate solid media with this liquid culture if necessary.
- (4) When not collected aseptically, place in a tube and process as sputum.

h. BLOOD

- (1) Add 10 ml aseptically collected blood to 50 ml of a Tween liquid medium such as Dubos or Middlebrook 7H-9.
- (2) Incubate at 35-37°C.
- (3) Smear once a week until acid-fast bacilli are found.
- (4) Then inoculate solid media with this liquid culture.

i. OTHER BODY FLUIDS

Muco-purulent materials:

- (1) Volume 10 ml or less; handle as for sputum.
- (2) Volume more than 10 ml; handle as for mucoid gastric lavage.

Fluid materials:

- (1) If collected aseptically, centrifuge and inoculate sediment directly onto culture media.
- (2) If not aseptically collected:
 - (a) Volume 10 ml or less; handle as for sputum.
 - (b) Volume more than 10 ml; handle as for fluid gastric lavage.

i. URINE

- (1) Centrifuge entire specimen for 30 minutes at 3000 rpm. (May need several tubes.)
- (2) Decant supernatant fluid and pool sediments.
- (3) Add an equal volume of 4% H₂SO₄ to sediment.
- (4) Mix and let stand for 15 minutes.
- (5) Fill the tube with sterile water.
- (6) Centrifuge. Decant supernatant fluid.
- (7) Add 0.2% bovine albumin to the sediment.
- (8) Inoculate media.

2. Incubation of Cultures

- a. Plates of inoculated media may stay for a few hours at room temperature before sealing in CO₂ permeable plastic bags for incubation. Keep shielded from light.
- b. Temperature at 35-37°C:

Cultures from skin lesion suspected to be *M. marinum* or *M.*

ulcerans must have a temperature not to exceed 33° C for primary incubation; however, incubate a duplicate culture at 37° C also.

c. Atmosphere: 5-10% carbon dioxide in air.

d. Medium in tubes:

Place in a slanted position with the screw-cap loose for at least one week to permit circulation of carbon dioxide for the initiation of growth. Tighten caps to prevent dehydration and stand tubes upright if space is a problem.

e. 7H-10 medium in plates:

Place plates medium-side down in a plastic bag; polyethylene allows carbon dioxide to pass into the bag. Seal the bag with tape, a heat-sealing apparatus, or fold over 2 or 3 times and staple. Squeeze the air out of the bag before sealing.

Stack not more than 6 plates in a pile.

To prevent sweating or the accumulation of drops of moisture in the plates during incubation, place plastic plates on an insulating surface, e.g., styrofoam, several thicknesses of cardboard, etc., and not directly on a metal shelf, tray, or basket.

3. Animal Inoculation

Guinea pig inoculation* may be profitable as an adjunct to culture with certain specimens, e.g.:

- a. Sputum from patients whose specimens are consistently contaminated on culture.
- b. Urine from patients with repeated negative cultures in whom tuberculosis is still suspected.
- c. Other body fluids where organisms may be few in number.

Generally, specimens for animal inoculation should be discouraged or referred to specialized laboratories. Multiple specimens and modern *in vitro* techniques are usually sufficient for isolation and identification.

All mycobacteria that produce disease in man do not produce progressive disease in the laboratory animal, e.g.:

- a. *M. tuberculosis* resistant to isoniazid.
- b. Clinically significant mycobacteria other than tubercle bacilli.

References:

Kubica, G.P., Dye, W.E., Cohn, M.L. and Middlebrook, G. 1963. Sputum digestion and decontamination with N-acetyl-L-cysteine-sodium hydroxide for culture of mycobacteria. Amer. Rev. Resp. Dis. 87: 775.

* INOCULUM: 1-2 ml of neutralized sediment, subcutaneously in the right groin of the guinea pig.

Kubica, G.P., Kaufmann, A.J. and Dye, W.E. 1964. Comments on the use of the new mucolytic agent, N-acetyl-L-cysteine, as a sputum digestant for the isolation of mycobacteria. *Amer. Rev. Resp. Dis.* 89: 284.

Corper, H.J. and Uyei, N. 1930. Oxalic acid as a reagent for isolating tubercle bacilli and a study of the growth of acid-fast nonpathogens on different mediums with their reactions to chemical reagents, *J. Lab. Clin. Med.* 15: 348.

Willis, H.S. and Cummings, M.M. 1952. *Diagnostic and Experimental Methods in Tuberculosis*, 2nd edition. Chas. C. Thomas, Springfield, Ill.

Meissner, G. 1964. The Bacteriology of the Tubercle Bacillus. *In* V.C. Barry (ed.) *Chemotherapy of Tuberculosis*, Butterworths, London.

4. Problem Areas

Adoption of new procedures frequently presents problems when a laboratory attempts to adapt them to the routine. These problems may stem from the omission of details in the published procedure and/or the failure to follow directions precisely. The major problem areas here appear to be: a. contamination, b. excess moisture in plates of 7H-10, c. poor growth on 7H-10 medium and, d. preparation and storage of media.

a. CONTAMINATION

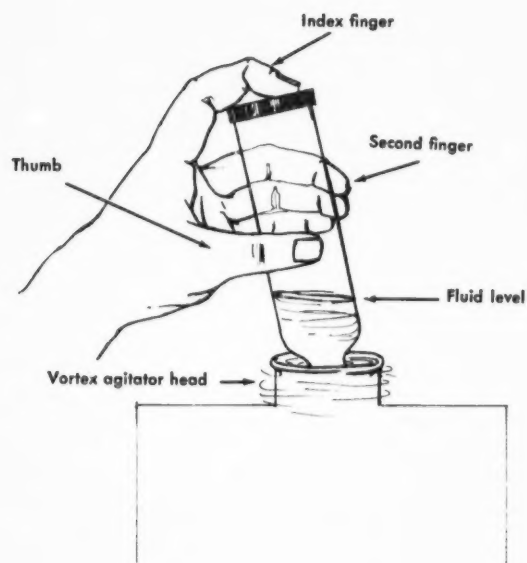
(1) Use the Vortex mixer properly (Figure 7).

Improper use may create a safety hazard caused by aerosol and leakage and/or increased rate of culture contamination. A description of the proper use of the Vortex mixer follows:

Add digestant-decontaminant (NALC) to specimen in the centrifuge tube. Tighten cap. Hold the tube *above* the fluid level and *below* the cap with thumb and three fingers. For increased stability the index finger may be placed on top of the cap. Holding tube at an angle, place it *gently* on the moving Vortex agitator-head and let the fluid swirl until liquified.

When the tube is held in this manner, the fluid will swirl no higher than the level of grip by fingers and thumb and will remain below the level of the cap.

If tube is incorrectly held, with thumb and fingers gripping near the cap, or if the tube is pressed too firmly on the Vortex mixer, fluid may swirl to the top and be retained on the inside of the cap. This retained fluid may not be decontaminated during the 15-minute decontamination period. Then, too, during centrifugation some of this fluid in the cap may leak down the outside of the tube, producing an aerosol in the centrifuge.



Proper Use of the Vortex Mixer

Figure 7

- (2) In digestion, perform the decontamination procedure properly.
 - (a) The NALC digestant used should not be more than 24 hours old. Oxygen combines with NALC, making it an ineffective digestant.
 - (b) Check to see if Na citrate was added. Na citrate complexes heavy metal ions which tend to bind the NALC, rendering it ineffective.
 - (c) If the specimen contains excess blood, the iron in the hemoglobin binds the NALC, making it impossible to digest the specimen.
 - (d) Mucoid materials should be completely digested. To aid mucolysis of "stubborn specimens," add a few extra crystals of N-acetyl-L-cysteine (10-30 mg) to the undigested specimen.
 - (e) More vigorous decontamination of digests may be needed. If so, increase the final concentration of NaOH

in digest from 2% to 3 or 4%. Do not increase decontamination time beyond 15 minutes. (Many mycobacteria are killed by over-decontamination.)

- (f) Follow directions precisely.
- (3) Check sterility of reagents and equipment.
- (4) Avoid air contaminants in the room.
 - (a) Check air flow in biological safety cabinet.
 - (b) Place ultra filters over air intakes.
 - (c) Swab floor and work surfaces with disinfectant.
 - (d) Consider UV irradiation of upper air and counter surfaces when the room is not in use.
- (5) Avoid contamination during incubation.
 - (a) Place plates with the medium side down in individual plastic bags. Seal with tape, rubber band, or staples. (This prevents the spread of contaminants and makes the early reading of results more likely.)
 - (b) If it is the practice to incubate plates in bulk in large plastic bags, stack the plates no more than six high. Do not handle the bags excessively, since opening and closing them to add or remove plates may introduce contaminants.
 - (c) Do not reuse plastic bags to incubate plates.
 - (d) Read plates first at 2 weeks. If earlier reading of a few cultures is necessary, use individual bags.
 - (e) Do not allow old cultures to remain in the incubator indefinitely.

b. EXCESS MOISTURE IN PLATES OF 7H-10

- (1) To avoid inoculating a wet medium surface, take plates out of the refrigerator on the day before they are to be inoculated and leave them at room temperature in a clean, dark area.
- (2) *After inoculation, shield plates from contaminating air currents and direct and indirect daylight.* Leave the plates for several hours before placing in the incubator. This permits some of the inoculum fluid to evaporate or be absorbed.
- (3) During incubation, follow these recommendations:
 - (a) Incubate plates in individual bags. This facilitates weekly reading of plates. Furthermore, moisture tends to accumulate in the plate on the bottom of the pile and rotation helps prevent this.
 - (b) If several plates are incubated in a large bag, stack no more than six high. Pressure of the upper plates may

"seal" moisture in lower plates and also interfere with the circulation of CO₂.

- (c) Incubate plastic plates on an "insulating" surface. Plastic plates in direct contact with metal shelves seem to collect more moisture than those "insulated" from the metal shelving. Styrofoam (1½ inch) is a satisfactory insulator.
 - (d) Avoid incubating plates on shelves adjacent to the door. Alternate warm and cool air currents created by opening and closing the door cause "sweating" and the collection of moisture within the plastic bags.
 - (e) Make sure that the air within the incubator is circulating properly.
- (4) When reading plates stacked in large plastic bags, open the bags immediately after removing them from the incubator. Plates incubated singly in transparent plastic bags may be read without opening the bag.

c. POOR GROWTH ON 7H-10 MEDIUM

- (1) Follow directions precisely in preparing the medium.
- (2) Plates should contain 20 ml; otherwise the medium may be too thin and dry out during storage or incubation.
- (3) Do not over-expose the medium to direct daylight during or after preparation.
- (4) Do not over-heat during preparation.
- (5) Make sure the supply of CO₂ in the incubator is sufficient.
 - (a) The recommended concentration of CO₂ is 5 to 10%. Concentrations above 40% may be toxic.
 - (b) A Fyrite CO₂ gauge, or some other means of determining the concentration of CO₂, should be used daily to test the atmosphere of the incubator.
 - (c) Be certain the plastic bags in which the plates are incubated are of the proper plastic to permit the circulation of CO₂. Polyethylene bags are permeable to CO₂; Mylar bags are *not*.
 - (d) Many strains of mycobacteria are dependent upon CO₂ and will not grow without it.
- (6) Over-decontamination may "damage" mycobacteria, thereby retarding their growth.

(7) Make sure there is no pH imbalance. Such an imbalance may be due to:

- (a) Failure to centrifuge sufficiently to pack the sediment so that all the supernate may be decanted, or
- (b) A too heavy inoculum, which interferes with the buffering action of the medium.

(8) Since some strains of mycobacteria grow more slowly than others and need an extra week or two of incubation to produce visible colonies, do not discard plates as negative until after at least six weeks.

d. PREPARATION AND STORAGE OF MEDIA

Preparation and storage problems are covered in Appendix E, pp. 112-118.

II. D. MICROSCOPY AND STAINING

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D. MICROSCOPY AND STAINING

1. Ziehl-Neelsen (Z-N)

a. REAGENTS

(1) Carbol fuchsin

(Prepare a saturated alcoholic solution by dissolving 3.0 g basic fuchsin in 100 ml of 95% ethyl alcohol.)

Saturated alcoholic basic fuchsin 10.0 ml
5% aqueous solution of phenol (liquefied crystals) 90.0 ml

(2) Acid alcohol

Ethyl alcohol (95%) 97.0 ml
Concentrated hydrochloric acid 3.0 ml

(3) Counterstain

Methylene blue chloride 0.3 g
Distilled water 100.0 ml

b. PROCEDURE

- (1) Prepare smear, using a 3-mm loop to spread a loopful over an area of 2-3 cm or smear a drop on the slide with a pipette.
- (2) Fix smears by gently heating over a Bunsen burner flame or on an electric slide warmer at 65° C to 75° C for at least 2 hours (overnight is acceptable).
- (3) Place a piece of filter paper over the smear. This holds the carbol fuchsin on the slide.
- (4) Cover the filter paper with carbol fuchsin and heat the slide to steaming with a Bunsen flame or an electric staining rack as follows:
 - (a) If a Bunsen flame is used, heat gently to steaming (DO NOT BOIL) and allow to stand 5 minutes without further heating. If the slide dries, keep it moist by adding more carbol fuchsin without additional heating.
 - (b) If an electric staining rack is used, place slides on the rack, turn on the switch, and allow to stain for 15 minutes. (If rack has been pre-heated, 7 to 10 minutes is sufficient.)

NOTE: Slides should be stained individually, since mass staining in a common container may allow cross transfer of acid-fast bacilli.

- (5) Remove filter paper strips and wash slides in tap water.
- (6) Decolorize with acid alcohol until no more color appears in the washings (about 2 minutes). Thick smears may require longer, but do not over-decolorize, since some mycobacteria may lose their acid-fastness if this is done.
- (7) Wash with distilled or tap water.
- (8) Flood slide with methylene blue counterstain for 1-2 minutes.
- (9) Wash with distilled or tap water.
- (10) Dry in the air or over gentle heat. Do not blot.
- (11) Examine smear with oil immersion lens, taking care to wipe the lens well after each examination, especially if the smear is positive. Three long lines the length of the smear or nine short lines the width of the smear are examined. The bacilli are stained red and the background material is stained blue. (See Figure 8.)

c. REPORTING SMEARS

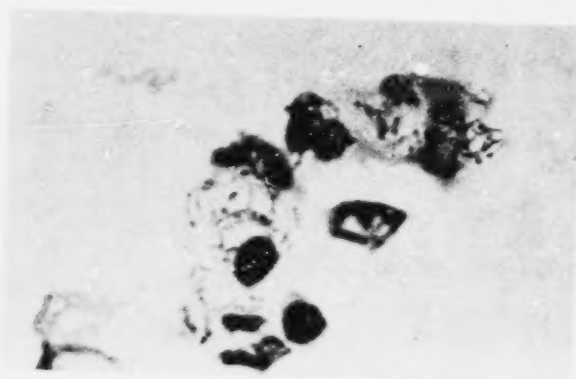
The National Tuberculosis Association recommends the following method:

Number of bacilli	Report
0	No acid-fast bacilli found.
1-2 in entire smear.	Report number found and request repeat specimen.
3-9 in entire smear.	Rare or +.
10 or more in entire smear.	Few or ++.
1 or more per field.	Numerous or +++.

References:

- National Tuberculosis Association. 1961, edition, Diagnostic Standards and Classification of Tuberculosis.
- Neelsen, —. Cited by Johne, —. 1885. Ein Zwifelloser Fall von congenitaler Tuberculose. Forstschr. d. Med. 3: 198.
- Ziehl, F. 1882, Zur farbung des tuberkelbazillus. Deutsch. Med. Wehnschr. 33: 451.

Figure 8 — Ziehl-Neelsen Stain of Acid-Fast Bacilli in Digested Sputum (about 1000 x magnification)



2. Fluorochrome

This stain employs dyes which fluoresce and which stain acid-fast organisms. No antigen-antibody reaction is involved. Therefore it is *not* and *should not be called* a fluorescent antibody test.

a. REAGENTS

(1) Stain

Auramine O	1.50 g
Rhodamine B	0.75 g
Glycerol	75.0 ml
Phenol (liquified crystalline phenol)	10.0 ml
Distilled water	50.0 ml

Combine and let stand overnight.

Filter through glass wool to clarify. This solution keeps several months at 4°C or room temperature. Store in a *dark bottle*.

(2) Decolorizing Solution (Acid-Alcohol)

0.5% HCl in 70% ethyl alcohol. (0.5 ml concentrated HCl in 100 ml 70% ethyl alcohol)

(3) Counterstain

0.5 g potassium permanganate in 100 ml distilled water. Store in a dark bottle.

b. PROCEDURE

- (1) Heat fix smears on slide warmer for at least 2 hours at 65°C-75°C (overnight is acceptable). Fixing on an electric staining rack is also acceptable.
- (2) Cover smear with auramine-rhodamine stain.
- (3) Stain for 15 minutes at room temperature (20-25°C).
- (4) Rinse with distilled or tap water.
- (5) Decolorize for 2-3 minutes with 0.5% HCl in ethyl alcohol.
- (6) Rinse with distilled or tap water.
- (7) Flood smear with 0.5% potassium permanganate "counter-stain." Allow to stand for not more than 2-4 minutes. Do not overcounterstain.
- (8) Rinse in distilled or tap water, air dry, and examine.

c. MICROSCOPIC EXAMINATION

- (1) Examine with fluorescence microscopy equipment and techniques. A BG-12 or 5113 primary filter with an OG-1 barrier filter system has proved very satisfactory.
- (2) With this system acid-fast organisms emit a bright yellow-orange fluorescence. Non-specific fluorescing debris is usually a pale yellow, quite distinct from the yellow-orange organisms.
- (3) Smears may be scanned rapidly with a 10X objective. Occasionally it may be necessary to use the 40X or 45X objective to ascertain actual bacterial morphology. (Figure 9 illustrates fluorochrome stained bacilli.)
- (4) Confirm all positive smears with a Ziehl-Neelsen stain. The fluorochrome smear may be overstained with Z-N after examination without removing the auramine-rhodamine stain. If overstained with Z-N, however, the smear is no longer satisfactory for fluorescent examination.

NOTE: Do not use fluorochrome stained smears to estimate inoculum size for drug susceptibility tests as described in this book. Reports should state that fluorochrome staining technique was used if smears are not confirmed by Ziehl-Neelsen.

d. REPORTING SMEARS

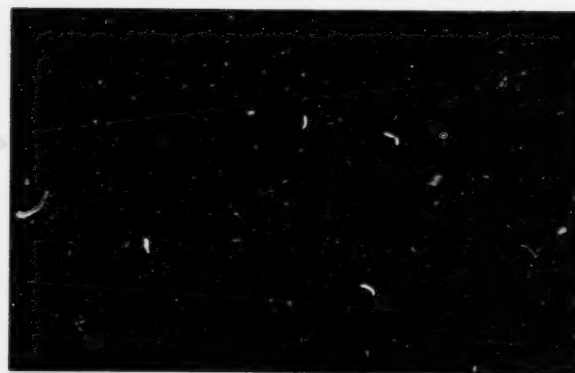
Two or more morphologically characteristic fluorescent bacilli must be seen before calling the smear positive. Otherwise report according to the National Tuberculosis Association method:

Number of bacilli	Report
3-9 per smear	Rare, +
10 or more per smear	Few, ++
1 or more per field	Numerous, +++

Figure 9 — Fluorochrome Staining of Acid-Fast Bacilli in Digested Sputum.



A. Observed at 100 x magnification



B. Observed at 450 x magnification

Reference:

Truant, J.P., Brett, W.A., and Thomas, W., Jr. 1962. Fluorescence microscopy of tubercle bacilli stained with auramine and rhodamine. *Bull. Henry Ford Hosp.* 10: 287.

NOTE: A fluorescence microscopy technique using an intense blue light instead of ultraviolet light has been devised. The authors list its advantages as: staining is quicker, cleaner, and simpler; the examination time can be reduced because only low power and high dry objectives are used; the greater contrast between the stained organism and the background reduces eye strain. This special lighting equipment and staining procedure are described by:

Morse, W. C., Blair, E. B. 1969. Fluorochrome staining and excitation of mycobacteria. From *Mycobact. Lab. Methods*. Report No. 317. U. S. Army Medical Research and Nutrition Laboratory, Denver, Colo. 80240.

3. Fluorescent Antibody Technique

The recognition that more than 90% of patients with newly diagnosed, never-before-treated cases of tuberculosis have organisms susceptible to SM, INH, and PAS has prompted many clinicians to institute treatment with these three drugs once a clinical diagnosis of tuberculosis has been established. A newly developed technique which might provide tentative bacteriologic confirmation of tuberculosis within 2 hours after receipt of the specimen is the fluorescent antibody (FA) method. At present, specific fluorescein-tagged antiserum (conjugate) must be prepared in those laboratories employing the technique. It is hoped, however, that a commercial supply of the conjugate may soon be available. Laboratories interested in trying the FA procedure should read the following:

Jones, W. D., Jr., Saito, H., and Kubica, G. P., 1965. Fluorescent antibody techniques with mycobacteria. *Am. Rev. Resp. Dis.* 92, No. 2, 255.

Jones, W. D., Jr., Beam, R. E., and Kubica, G. P., 1967. Fluorescent antibody techniques with mycobacteria II. Detection of *Mycobacterium tuberculosis* in sputum. *Am. Rev. Resp. Dis.* 95, No. 3, 516.

Jones, W. D., Jr., Kubica, G. P., 1968. Fluorescent antibody techniques with mycobacteria III. Investigations of five serologically homogeneous groups of mycobacteria. *Zen. Bakt.* 207, 58.

II. E. CULTURE READING AND IDENTIFICATION

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E. CULTURE READING AND IDENTIFICATION

1. Introduction

Mycobacterium tuberculosis is the most common species of mycobacteria isolated from patients suspected of having tuberculosis. Acid-fast bacilli other than *M. tuberculosis* may also occur in patient specimens. Some of these bacilli may be implicated in disease. It is therefore imperative that the *M. tuberculosis* be identified as such and that the potential clinical significance of the other mycobacteria be determined.

Many of the mycobacteria in the Runyon Groups have been given species status and, consequently, species names. In most cases, it is possible to use legitimate names rather than the indefinite Runyon Group classification. The use of species names will assure that only one label is applied to a given species of organism, thus preventing confusion. See Table I.

2. Culture Reading

Careful attention to detail yields valuable information for identification of cultures. A uniform method of recording and filing laboratory data is essential for culture identification and serves as a safeguard when questions arise concerning a report. Cultures represent people; therefore, accuracy is imperative.

a. METHOD

For reading cultures in Lowenstein tubes, use 3-10X hand lens or other magnifier.

For 7H-10 invert the plates on the stage of the dissecting microscope. Read at 10-60X with transmitted light. Scan rapidly at 10-20X for the presence of colonies. Higher magnification (30-60X) is helpful in observing colony morphology.

b. TIME

Read all cultures within 5 to 7 days after inoculation and once a week thereafter.

Lowenstein-Jensen Medium

Discard as negative after 6 to 8 weeks.

7H-10 Medium

When plates are sealed in individual bags, read within 5 to 7 days after inoculation and once a week thereafter for 6 weeks.

When several plates are sealed in one bag, read first after 2 or 3 weeks of incubation. Reincubate negative and doubtful cultures in a fresh plastic bag. Read after 4 weeks and again after 6 weeks.

TABLE I
Potential Clinical Significance of Various Mycobacteria

Runyon Group	Original Definition	Always or Sometimes Significant	Never or Rarely Significant
I	Slowly growing photochromogens. Pigmentation occurs only under the influence of light	<i>M. tuberculosis</i> <i>M. bovis</i>	
		<i>M. kansasii</i> <i>M. marinum (balnei)</i>	<i>M. kansasii</i> (low catalase variety)
II	Slowly growing scotochromogens. Definitely pigmented yellow-orange even if grown in the dark. Often more deeply colored if grown exposed to light.	<i>M. scrofulaceum</i>	Tap water scotochromogen <i>M. flavescens</i> *
III	Slowly growing nonphotochromogens. Usually non- or weakly pigmented. Old cultures or cultures that have been transferred many times may be scotochromogenic.	<i>M. avium</i> <i>M. intracellulare (Battey)</i> <i>M. xenopi</i>	<i>M. gastri</i> ("J") <i>M. terrae</i> complex (includes <i>M. nonchromogenicum</i>) <i>M. triviale</i> ("V")
IV	Rapidly growing mycobacteria	<i>M. fortuitum</i>	<i>M. smegmatis</i> <i>M. phlei</i> <i>M. vaccae</i> <i>M. fortuitense</i>

* This organism is intermediate in speed of growth and may be considered either a slow or a rapid grower. Most identification tests, however, tend to place *M. flavescens* in the rapidly growing mycobacteria.

Note: Never discard a culture as negative without first observing under magnification for minute colonies.

c. OBSERVATIONS TO RECORD

- (1) Note the number of days required for colonies to become macroscopically visible (record date).

- (2) Number of colonies is recorded as follows:

No colonies	=	Negative	
Less than 50 colonies	=	Actual Count	
50-100 colonies	=	+	(1+)
100-200 colonies	=	++	(2+)
Almost confluent (200-500)	=	+++	(3+)
Confluent (more than 500)	=	++++	(4+)

- (3) Pigment production may be observed on egg-base or 7H-10 medium. It may be recorded as follows:

White, cream, or buff	=	Nonchromogenic (NC)
Lemon yellow, orange, red	=	Chromogenic (Ch)

Intermediate colorations such as pink, pale yellow, or tan sometimes occur. These are recorded as observed and not as chromogenic. These cultures are regarded as nonphotochromogens unless the pigment becomes more intense upon light exposure.

- (a) Light induced pigment as seen with photochromogens: Colonies are nonchromogenic when incubated in the dark, but after they are exposed to light for 1 hour and reincubated, they become chromogenic (Figure 10).
- (b) Scotochromogens are those cultures whose colonies are chromogenic when grown in the dark. When the cultures are exposed to light continuously for 2 weeks, the pigment in the colonies may deepen to orange or red (Figure 11).
- (c) Nonphotochromogens are those cultures whose pigment is not changed by exposure to light for 2 weeks.
- (d) Absorbed pigment or the "greening" phenomenon may be seen when rare strains of *M. fortuitum* or *M. tuberculosis* appear to absorb the malachite green from egg medium.

- (4) Colony morphology is best observed with isolated colonies. Colonies are usually described as rough, smooth, domed, etc. For examples of rough and smooth colony types on Lowenstein-Jensen egg medium, see Figures 12 - 14.

Colony variations on the transparent 7H-10 agar medium may be observed with the aid of a dissecting microscope, see Figure 15.

Under magnification young developing colonies are seen as clusters of bacilli that develop into typical colony forms as they mature.

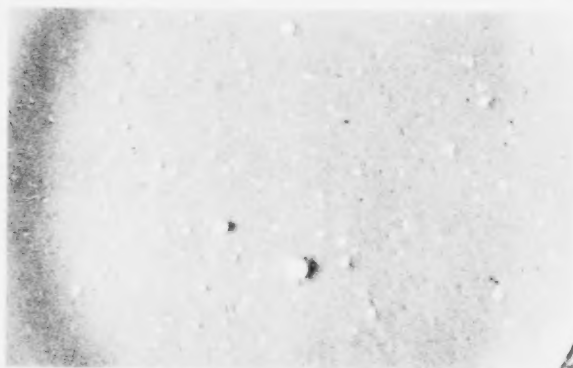
Convenient symbols for recording colony characteristics are:

RgR = rough	I = interconvertible (mixture of Sm and D)
Sm = smooth	NC = non-chromogenic
D = domed (hemispherical)	y = yellow
S = spreading	o = orange
T = transparent	s = spot in center
X = xenopi	a = apron (thin growth at the base of the colony)
K = kansasii	f = filamentous.
"K" = resembles kansasii	w = wrinkled (not rough, cords)

A combination of descriptive symbols may be used, such as:

RgRy = rough yellow	SmDa = smooth domed with apron
SmS = smooth spreading	SmI = a mixture of SmS and SmD
SmSy = smooth spreading, yellow	SmT = smooth transparent (thin, spreading)

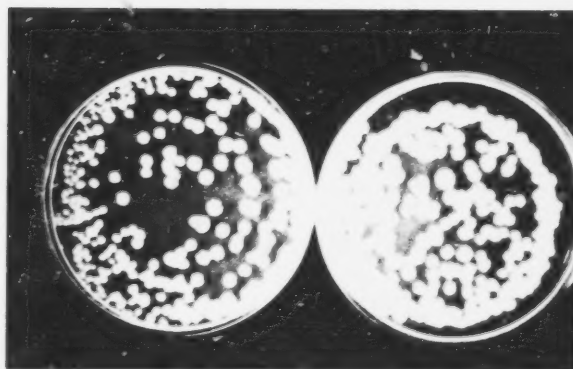
Figure 10 — Pigment Formation in Strains of *M. kansasii* Exposed to Light



A. Rough Strain on egg medium, grown in dark

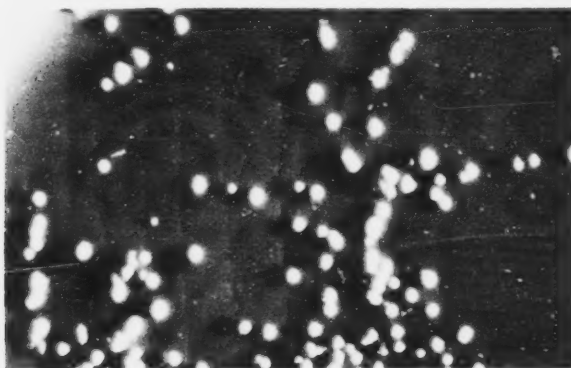


B. Same Strain after exposure to light

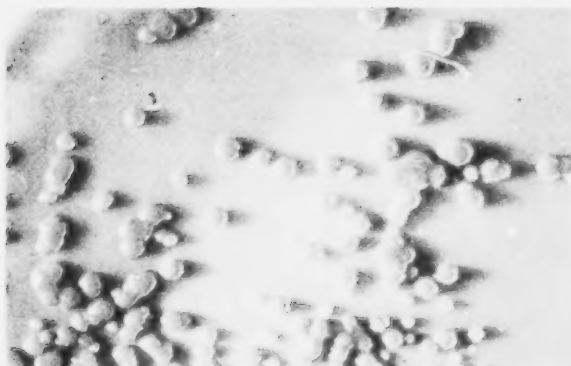


C. *M. kansasii* on 7H-10 plates before and after light exposure

Figure 11 — Intensification of Pigment in Scotochromogen by Light Exposure



A. Grown in the dark

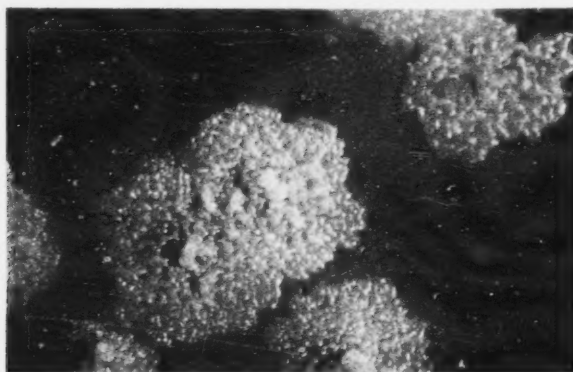


B. Exposed to light for 2 weeks

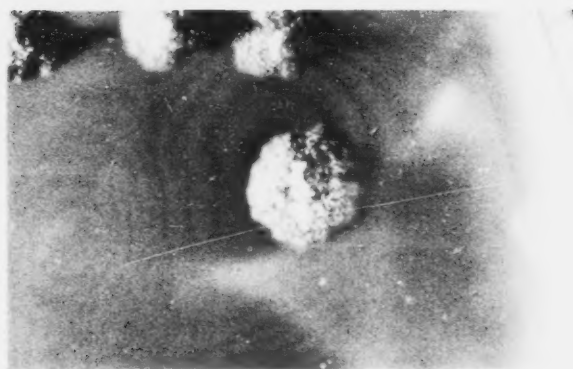


Figure 12 — Smooth Strain of *M. fortuitum* on Lowenstein-Jensen Egg Medium

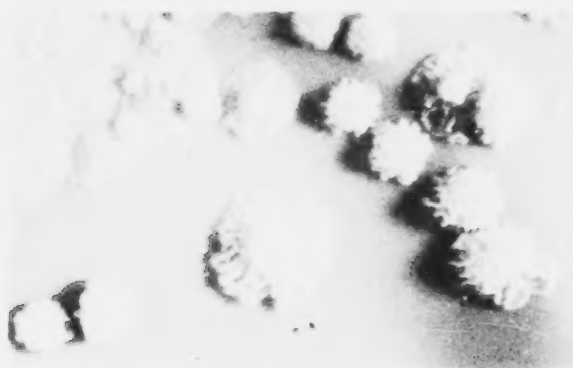
Figure 13 — Examples of Rough Colony Types Observed on Lowenstein-Jensen Egg Medium



A. *M. tuberculosis*

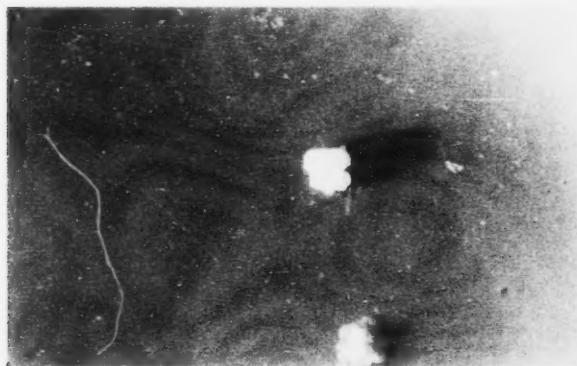


B. *M. fortuitum*



C. *M. smegmatis*

Figure 14 — Other Colony Types Seen on Lowenstein-Jensen Egg Medium



A. *M. kansasii*, rough colony

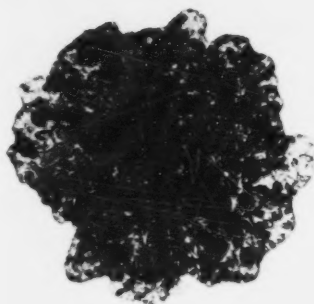


B. Close-up of translucent, pyramid-shaped colony of *M. bovis*

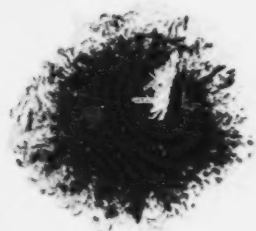


C. Nonphotochromogen (Battey)

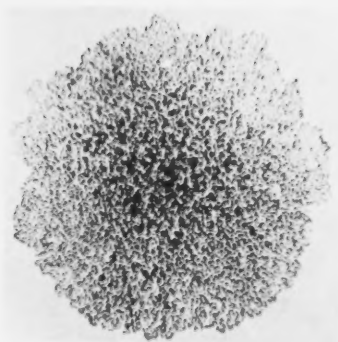
Figure 15 — Colonies on 7H-10 Agar Medium



Rough R (RgR), dry, white, buff. Made up of serpentine cords with a heaped-up center. Mature in 10-20 days. Seen in *M. tuberculosis*. *M. bovis* resembles this colony, but matures more slowly.



Rough R (RgR), white, buff, or yellow. Dense, coarse, texture. Mature in 5-7 days. Rapid growers such as *M. fortuitum*, *M. phlei*, *M. smegmatis*, others.

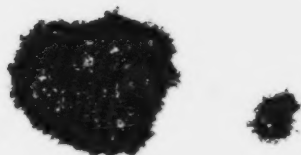


Rough R, white, buff, or yellow. Mature in 10-14 days. Seen in *M. kansasii*, also in scotochromogens and non-chromogens such as *M. triviale*.

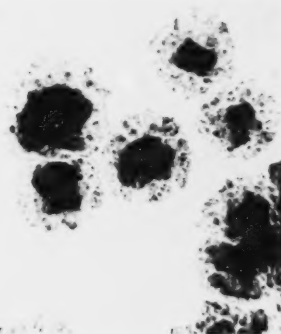


Rough R, wrinkled as opposed to cords. Mature in 10-14 days. Seen in *M. kansasii* more often than others.

Figure 15—Continued



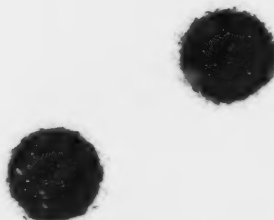
Rough X or Smooth X. Tiny domed colonies with short stick-like projections. Surface of dome may be rough or smooth. Usually yellow at maturity. Requires 21 to 28 days for minute colonies. Typical *M. xenopi*.



Smooth Kw or K. Non-chromogenic in the dark and yellow in the light. With transmitted light has a characteristic sheen. Often wrinkled as opposed to cords. Mature in 10-14 days. Typical *M. kansasii*.

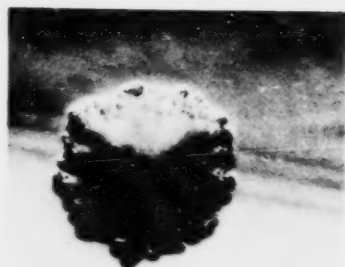


Smooth Domed (SmD). Hemispherical colonies. White, cream, yellow. Mature in 10-14 days. Seen in non-photochromogens, scotchromogens, and rapid growers. Often have granules in center.

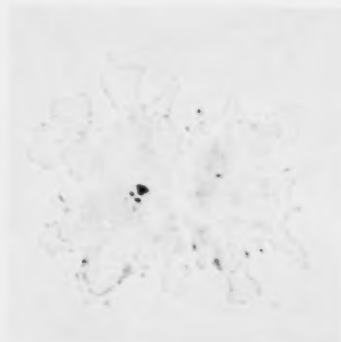


Smooth Domed with apron of growth at base of colony (SmDa). Seen in rapid and slow growers, such as nonphotochromogens and scotchromogens. White, cream, or yellow.

Figure 15—Continued



Smooth Spreading (SmS). Raised center, the outer area becoming thin with an undulating edge. White, cream, yellow. Mature in 10-14 days. Seen in nonphotochromogens and scotochromogens.



Smooth transparent (SmT). Like the SmS colony, but thin with only slightly raised center. White, cream, yellow. Mature in 10-14 days. Seen in nonphotochromogens and scotochromogens.

References:

- Fregnan, G. C. and Smith, D. W. 1962. Description of various colony forms of mycobacteria. *J. Bact.* 83: 819
- Kubica, G. P. and Jones, W. D. 1965. Differential colonial characteristics of mycobacteria on oleic acid-albumin and modified corn meal agars. I. Investigation of slowly growing mycobacteria. *Zentralblatt für Bakt.* 196: 53
- Jones, W. D. and Kubica, G. P. 1965. Differential colonial characteristics of mycobacteria on oleic acid-albumin and modified corn meal agars. II. Investigation of rapidly growing mycobacteria. *Zentralblatt für Bakt.* 196: 68
- Pattyn, S. R. 1966. A study of some strains of *Mycobacterium xenopi*. *Zbl. Bakt.* 1 Orig. 201 3: 246-252
- Runyon, E. H. 1968. Aerial Hyphae of *Mycobacterium xenopi*. *J. Bact.* 95: 734-735
- Vestal, A. L. and Kubica, G. P. 1966. Differential colonial characteristics of mycobacteria on Middlebrook and Cohen 7H-10 agar-base medium. *Am. Rev. Resp. Dis.* 94: 247.
- Wayne, L. G., Runyon, E. H., Kubica, G. P. 1969. Mycobacteria: a guide to nomenclature usage. *Am. Rev. Resp. Dis.* (submitted for publication).

3. Culture Identification

Make a smear of growth on the culture to determine its acid-fastness and purity.

Separate cultures according to growth rate:

RAPID GROWERS are fully matured within 7 days (on subculture).

SLOW GROWERS require more than 7 days.

When in doubt about growth rate, make a subculture with small inoculum to secure isolated colonies. Primary isolates must adapt to an artificial environment; therefore, they may grow more slowly than a subculture.

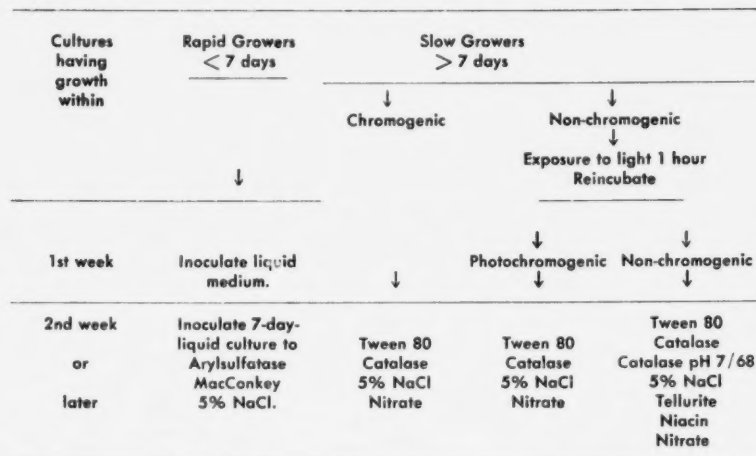
One quadrant or slant of medium with at least 100 colonies is a sufficient amount of growth for performing several identification tests.

Test or inoculate as follows (see page 45 for work-flow chart):

Culture	Test or Inoculate	Page
Rapid Grower	Transfer to liquid medium*	
	for inocula for arylsulfatase.....	65
	and MacConkey	68
	5% NaCl	70
All Slow Growers (chromogenic and non-chromogenic)	Inoculate Tween 80.....	58
	Catalase R°	60
	Nitrate reduction	55
	5% NaCl	70
All Non-chromogenic Slow Growers	Expose to 1 hour light.....	68
	Catalase pH 7/68°C.....	62
	Tellurite test	64
	Niacin test	53
Mixed Cultures	Separate and identify	74
Contaminated	Purify and identify.....	73
Insufficient growth	Transfer to liquid medium* and/or solid medium	
Suspected <i>M. bovis</i>	Inoculate a rabbit.....	71
	Inoculate TCH	72

* Dubos Tween-albumin or Middlebrook 7H-9.

**Work-Flow Chart for Identification
of Mycobacterium Cultures**



TIMETABLE FOR COMPLETING IDENTIFICATION TESTS

One-hour light exposure: reincubate. Read in 8-24 hours.
 Arylsulfatase: incubate 3 days, add Na carbonate. Read.
 Tellurite: add tellurite to 7-day culture. Incubate 3 days. Read.
 Tween 80 hydrolysis: incubate. Read at 5 and 10 days.
 MacConkey agar: incubate. Read at 5 and 11 days.
 Catalase: when growth is adequate, perform the tests.
 Niacin test: perform on culture 3 to 4 weeks old.
 Nitrate reduction: perform on culture 3 to 4 weeks old.
 5% NaCl: read once a week for 4 weeks.

When results of identification tests do not conform to the usual results for classifying an organism, repeat the test or tests at least once. Variations may be due to: (1) *contamination*, (2) *mixed cultures*, (3) *biological variation*, and/or (4) *environmental influences*.

If after repeating the test, the organism still does not fit a known species pattern, refer the culture to a specialty laboratory, e.g., a State health department laboratory or the NCDC.

a. RAPID GROWERS

Mature colonies are visible without magnification in less than 7 days. The species *M. fortuitum* is the potential pathogen in this group and should be so identified.

M. abscessus, an organism whose species status is not yet firmly established, is closely related to *M. fortuitum* and gives the same reactions as *M. fortuitum* in the tests described. Since *M. abscessus* can produce pathology in man, there should be no reason for concern if it is incorrectly identified as *M. fortuitum*.

Other species of rapid growers such as *M. vaccae*, *M. borstelense*, *M. smegmatis*, and *M. phlei* are considered saprophytes and require special tests for specific identification.

M. fortuitum may be separated from other rapid growers by MacConkey agar and arylsulfatase activity.

(1) Colony characteristics:

On Lowenstein-Jensen or 7H-10 medium the colonies may be rough or smooth.

On 7H-10 the Rough R forms seem to be composed of coarse cords; the smooth forms are very opaque and dense but slope off near the periphery of the colony, allowing light to penetrate the irregular outer edge. Other rapid growers produce a variety of dense opaque, patterned colony forms.

(2) Pigment production

Most strains are nonchromogenic; others may be yellow, orange, or pink.

(3) "Greening" on egg medium

M. fortuitum colonies may apparently absorb malachite green.

(4) Summary of characteristics:

Species	Colonies 7H-10	Colonies L-J	Pigment	NaCl	Arylsulfatase*		Mac- Conkey
					3 day	2 week	
<i>M. fortuitum</i>	Rg or Sm	Rg or Sm	NC and "greening"	+	$\pm/2+$	3/5+	+
Rapid Growers	Rg or Sm	Rg or Sm	NC or Y	+	-/1+	-/3+	-
<i>M. phlei</i>	Rg or Sm	Rg	Y	+	-	-/3+	-
<i>M. smegmatis</i>	Rg or Sm	Rg	NC	+	-	3+	-

*When results are separated by a slash, as in $\pm/2+$, the variations in test reactions, low/high, are indicated.

b. NONCHROMOGENIC SLOW GROWERS

Mature colonies require more than 7 days to be visible without magnification. Their color varies from white to buff.

- (1) *M. tuberculosis* is the mycobacterium in this group most commonly isolated from clinical disease. Non-chromogenic rough colonies that are niacin positive and drug sensitive usually are *M. tuberculosis*.

Niacin negatives should be repeated on a culture with "older" growth, especially if the culture is nitrate positive and pH 7/68°C catalase negative. Drug resistant strains of *M. tuberculosis* may be catalase negative and have smooth colony forms on Lowenstein-Jensen. Colonies on 7H-10 are consistently rough.

- (2) *M. bovis* rarely occurs in man in the U.S.A. but it may be isolated from clinical specimens. Colonies on Lowenstein are tiny, smooth, pyramidal, and translucent, often resembling the surface of the medium. On 7H-10 the colonies are always rough, resembling *M. tuberculosis*, but are usually niacin negative. This species, the least reactive in the tests, gives a positive result only in the room temperature and semi-quantative catalase tests. It may be separated from *M. xenopi*, *M. gastri*, and *M. avium* - *M. intracellulare* by the pH7/68°C catalase, Tween 80, and tellurite tests. Final identification should be confirmed by rabbit inoculation and/or sensitivity to thiophen-2-carboxylic acid hydrazide (TCH).

- (3) *M. avium* causes tuberculosis in fowl, swine, and occasionally in other animals, but rarely in man. *M. intracellulare* produces mycobacterioses in man and some lower animals. With the limited number of tests given here this pair of organisms cannot be separated. Treatment of disease is similar because both strains are relatively drug resistant.

M. avium - *M. intracellulare* cultures usually have predominately Smooth S, T, or D colony forms on primary isolation and produce an increasing number of rough variants on transfer. More than one colony form may be found within the same culture, and strains show varying shades of pale pigment as they mature. Identification tests are necessary to separate these strains from the other

nonphotochromogens. These organisms are clinically significant and may be recognized by a positive tellurite reduction test in 3 days, a negative Tween 80 hydrolysis at 10 days, and negative niacin and negative nitrate reduction tests.

- (4) *M. xenopi* may be implicated in human disease. It grows very slowly, usually requiring 4 to 5 weeks for the tiny domed colonies to become visible. With magnification the colony on 7H-10 medium resembles a "miniature bird's nest" with a rough or smooth dome having short stick-like projections visible at the periphery of the colony. These domed colonies have been labeled "Rough X" or "Smooth X" because of the unique appearance. Mature colonies often are pale yellow. Colony morphology, growth rate, and the identification test results are usually sufficient to separate them from other slow growers.
- (5) *M. kansasii* is nonchromogenic when grown in the dark. It characteristically has a Smooth K colony on 7H-10; a few rough strains may be encountered and mis-typed as *M. tuberculosis* or another rough nonchromogenic mycobacterium unless the light exposure test is properly performed. Traces of yellow pigment, undetected by casual observation, may be extracted with the niacin reagents producing a false-positive test result. As a help in avoiding errors in typing, its characteristics are included in this summary table.
- (6) Other nonphotochromogens which are usually not pathogenic are:
 - M. terrae* complex: colonies may be rough or smooth. This complex includes the closely related organisms *M. nonchromogenicum*, *M. terrae*, and possibly others as yet not identified. These organisms are commonly found in the soil and water and subsequently in clinical specimens.
 - M. gastri* ("J"): colonies resemble Smooth "K" on 7H-10 except the central spot is smaller. These have been isolated from gastric washings.
 - M. triviale* ("V"): thin rough colonies on 7H-10, usually rough on Lowenstein. These may be isolated from clinical specimens.

(7) A Summary of Characteristics

Species	Colony Characteristics		1 hr. light	Catalase Activity*					Tellurite Reduction in 3 days	Growth on 5% NaCl	
	7H10	L-J		Niacin	Nitrate Reduction	R°	pH7 68°	Tween hydrolysis			
POTENTIAL PATHOGENS											
<i>M. tuberculosis</i>	RgR	Rg	-	+	3/5+	+	-	-/+	-	-	
<i>M. bovis</i>	RgR	Sm	-	-	-	+	-	-	-	-	
<i>M. kansasii</i>	K or RgR	Sm or Rg	+	-	3/5+	+	+	+	-	-	
<i>M. xenopi</i>	SmX-RgX	Sm	-	-	-	+	+	-	-	-	
<i>M. avium</i>	SmI-RgR	Sm or Rg	-	-	-	+	+	-	-	-	
<i>M. intracellulare</i>	SmI-RgR	Sm or Rg	-	-	-	+	+	-	+	-	
NON-PATHOGENS											
<i>M. terrae</i> complex	SmI-RgR	Sm or Rg	-	-	±/4+	+	+	+	-	-	
<i>M. gastri</i> ("J")	"K"-like	Sm	-	-	-	+	-	+	-	-	
<i>M. triviale</i> ("V")	RgR	Rg	-	-	3/5+	+	+	+	-	+	

*Reaction ++ = rapid, + = slow.

*Reaction ++ = rapid, + = slow.

c. CHROMOGENIC SLOW GROWERS (SCOTOCHROMOGENS)

The potential pathogen in this group is *M. scrofulaceum* and should be identified as such. The tap water scotochromogens of this group are usually not associated with disease.

(1) Colony characteristics:

Smooth with domed or spreading colonies having a definite yellow pigment even when grown in the dark. The scotochromogens have a wide variety of colony forms: Smooth Sy, Dy, Ty, or an occasional Rough Ry.

(2) Effect of light on pigment:

When exposed to "continuous" light for 2 weeks, many strains increase in pigment from yellow to orange or brick red. If cultures are exposed to continuous light, they should be incubated in the dark until visible colonies appear and then placed in continuous light. Initial growth may be inhibited by too much light.

(3) *M. flavescens* is intermediate in speed of growth and may be considered either a rapid or slow grower. Most identification tests, however, tend to place it among the rapidly growing mycobacteria.

(4) Chromogenic cultures that have been exposed to light in the incubator or intermittently during weekly culture reading should be subcultured for the light exposure test. Unless they are kept in the dark during incubation, photochromogens may be mis-typed as scotochromogens.

(5) The Tween 80 test separates the clinically significant *M. scrofulaceum* from others of this group.

(6) Summary of Characteristics:

Species	Colonies		Pigment		1 hr. exp.	NO ₃	Tween 80	NaCl
	7H-10	L-J	dark	light				
<i>M. scrofulaceum</i>	Sml or Rg	Sm or Rg	y	y-o	—	—	—	—
Tap Water	Sml or Rg	Sm or Rg	y	y-o	—	—	+	—
<i>M. flavescens</i>	Sm or Rg	Sm or Rg	y	y-o	—	+	+	±
<i>M. kansasii</i>	K or Rg	Sm or Rg	NC	y-o	y	+	+	—

d. PHOTOCHROMOGENS

The unique characteristic of the photochromogens is that the colonies are nonchromogenic when grown in the dark but when exposed to light for 1 hour and reincubated for 6-24 hours, they become chromogenic.

Strains of *M. kansasii* with high catalase activity have been associated with disease more often than those strains with low catalase activity.

- (1) *M. kansasii* colonies on 7H-10 are Smooth K or Kw. Rough strains also occur.
- (2) Cultures with confluent growth or old colonies may not produce pigment after exposure to light for 1 hour. Careful light studies and observation of colony characteristics are important, since identification test results are similar to those of clinically insignificant mycobacteria.
- (3) Exposure to continuous light for 2 weeks during incubation often produces orange crystals within isolated colonies. These are characteristic of *M. kansasii*.
- (4) *M. marinum* is also a photochromogen. Cultures of specimens from skin lesions should be incubated at 33°C. and 37°C. On primary isolation some strains may grow only at 33°C; others, at 27°C.

(5) Summary of Characteristics:

Species	Colonies		Niacin	NO ₂	Catalase Semiquan. mm	Arylsulfatase*	
	7H-10	L-J				3 da.	2 wk.
<i>M. kansasii</i>	K or Rg	Sm or Rg	—	+	< 40 or > 50	—	—/1+
<i>M. marinum</i>	"K"	Sm	— or +	—	< 40	—/2+	4+

* Perform only when other tests indicate *M. marinum* or send culture to a reference laboratory.

Table II
4. SUMMARY TABLE: GROWTH CHARACTERISTICS AND IDENTIFICATION OF MYCORACTERIA*

Species	Growth in less than 7 days	Pigment formation		Nicotin	Nitrate Reduction		Catalase Activity		Tween hydrolysis (days)			Tellurite Reduction in 3 days	Growth on 5% NaCl	Arylsulfatase		Growth on MacCon- key agar
		Dark	Light		1 hour	pH7 68°	Semiquan.		5	10	3			2 week		
							<40 mm	>50 mm								
<i>M. tuberculosis</i>	—	—	—	+	+	+	+	—	±	+	—	—	—	—	—	—
<i>M. bovis</i>	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
<i>M. kansasii</i>	—	—	+	—	+	+	+	+	+	+	—	—	—	—	—	—
<i>M. marinum</i>	—	—	+	—	+	±	±	+	+	+	—	—	—	—	—	—
<i>M. scrofulaceum</i>	—	+	+	—	—	—	—	+	—	—	—	—	—	—	—	—
Tap Water	—	+	+	—	—	—	—	+	+	+	—	—	—	—	—	—
<i>M. flavescens</i>	±	+	+	—	+	+	+	+	+	+	—	—	±	—	—	—
<i>M. avium</i> **	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
<i>M. intracellulare</i> **	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—
<i>M. xenopi</i>	—	±	±	—	—	—	—	+	—	—	—	—	—	±	2/5+	—
<i>M. gastri</i> ("J")	—	—	—	—	—	—	—	+	+	+	—	—	—	—	—	—
<i>M. intras complex</i>	—	—	—	—	+	+	+	+	+	+	—	—	—	—	—	—
<i>M. triviale</i> ("V")	—	—	—	—	+	+	+	+	+	+	—	—	+	±	3/5+	—
<i>M. fortitum</i>	+	—	—	—	±	±	±	+	±	±	—	—	—	±/2+	3/5+	+
<i>M. smagmatis</i>	+	—	—	—	±	±	±	+	+	+	—	—	—	—	3+	—
<i>M. phlei</i> **	+	+	+	—	+	+	+	+	+	+	—	—	—	—	—	—
<i>M. vaccae</i> **	+	+	+	—	+	+	+	+	±	+	+	+	+	—	3+	—
<i>M. borstelense</i>	+	—	—	Y	—	—	—	+	—	—	—	—	—	±/2+	3+	±

* Variations are to be expected.

** With tests listed, the pairs of organisms so indicated cannot be separated; colonial morphology on 7H-10 may be helpful in the case of *M. phlei*—*M. vaccae*.

Key to symbols:

— = usually negative

± = negative, rarely positive

± = positive, rarely negative

+ = usually positive

/ = low to high variations

V = variable; niacin + species is *M. borstelense* var. *niacinogenes*

II. F. IDENTIFICATION TEST TECHNIQUES

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F. IDENTIFICATION TEST TECHNIQUES

1. Niacin Test

a. CULTURES

Test all nonchromogenic mycobacteria for niacin production. (From 7H-10 medium,* only the rough nonchromogenic strains need to be tested for niacin.) A culture must have at least 50-100 colonies with growth 3 to 4 weeks old. *M. tuberculosis* is usually niacin positive. Most other mycobacteria are niacin negative.

b. EQUIPMENT

Sterile screw-cap test tubes, 16 x 125 mm. Label with numbers to correspond with cultures to be tested.

Sterile pipettes, 1 ml and 5 ml

c. REAGENTS

Sterile distilled water or 0.85% saline.

4% aniline

To 96 ml of 95% ethyl alcohol, add 4.0 ml colorless aniline. Store in a brown bottle in the refrigerator. If the solution turns yellow, discard and prepare fresh solution.

10% cyanogen bromide

Dissolve 5 g cyanogen bromide in 50.0 ml distilled water. Store in a tightly capped brown bottle in the refrigerator. If a precipitate forms upon cooling, warm to room temperature to dissolve. Prepare in small quantities because the reagent is volatile and loses strength. Weak solutions may give false negative readings.

CAUTION: Cyanogen bromide is tear gas and should be used in a well-ventilated safety cabinet. Cyanogen bromide also may hydrolyze in acid solution to hydrocyanic acid; discard niacin tests into a germicidal solution made alkaline by the addition of NaOH.

d. CONTROLS

Reagent control on uninoculated medium (negative).

Reagent control on *M. intracellulare* (negative).

Reagent control on *M. tuberculosis* culture (positive).

* The niacin test from cultures on 7H-10 medium is satisfactory when the 7H-10 contains 0.1% aspartic acid.

e. PROCEDURE

- (1) To the culture slant or quadrant on drug-free medium, add 1.0 ml sterile distilled water or saline.
- (2) If growth on the medium is completely confluent, puncture the surface so the liquid is in contact with the medium. Niacin, if present, is extracted from the medium, not from the bacterial growth.
- (3) Allow the fluid to remain in contact with the culture medium for 15 minutes. When the culture is in a tube, place in a slanted position.
- (4) Remove 0.5 ml of the liquid extract from the culture and place in a numbered clean screw-cap test tube.
- (5) Add 0.5 ml of aniline (equal volume) to the extract. This should be colorless.
- (6) Next, add an equal volume (0.5 ml) of cyanogen bromide.
- (7) Observe for a yellow color which should appear immediately.

f. RESULTS

Positive = yellow color.

Negative = no color change.

For examples, see Figure 16.



Figure 16 — Niacin Test

References:

- Konno, K. 1956. New chemical method to differentiate human-type tubercle bacilli from other mycobacteria. *Science* 124: 985.
- Runyon, E. H., Selin, M. J. and Harris, H. W. 1959. Distinguishing mycobacteria by the niacin test. *Am. Rev. Resp. Dis.* 79:663.

2. Nitrate Reduction Test

a. CULTURES

Test all nonchromogenic cultures 3 to 4 weeks after visible colonies appear. *M. tuberculosis*, *M. kansasii* are the clinically significant nitrate positives.

Of the chromogenic cultures, *M. flavescens* is nitrate positive whereas the slow growing scotochromogens are negative.

b. EQUIPMENT

Screw-cap test tubes (16 x 125). Label with numbers to correspond to the cultures to be tested. Label tubes for controls.

Sterile pipettes, capillary or 1 ml and 5 ml.

Inoculating spade or loop.

Water bath at 37°C or constant temperature block heater.

c. REAGENTS

Substrate: M 100 sodium nitrate in M/45 phosphate buffer prepared as follows:

NaNO ₃	0.085 g
KH ₂ PO ₄	0.117 g
Na ₂ HPO ₄ · 12H ₂ O	0.485 g
Distilled water	100.0 ml

Test reagents:

No. 1 A 1:2 dilution of HCl (add 10 ml HCl to 10 ml H₂O).

No. 2 Dissolve 0.2 g sulfanilamide in 100 ml H₂O.

No. 3 Dissolve 0.1 g N-naphthylethylenediamine dihydrochloride in 100 ml distilled water.

Store reagents and substrate in the dark in the refrigerator. If precipitate forms or if reagents No. 2 and No. 3 change color, discard and prepare fresh solutions.

Distilled water, sterile.

Color standard for reading test (Appendix page 110).

d. CONTROLS

Reagent control without organisms (negative).

Reagent control nitrate positive *M. tuberculosis* (positive).

e. PROCEDURE

(1) Place 3 or 4 drops of sterile distilled water in each sterile screw-cap test tube.

(2) Emulsify in the water one loopful or spadeful of colonies from solid medium.

- (3) Add 2 ml of the NaNO_3 substrate solution to each test.
- (4) Shake by hand to mix and incubate at 37°C for two hours. Remove from water bath.
- (5) Add to each tube 1 drop of Reagent No. 1.
- (6) Add to each tube 2 drops of Reagent No. 2.
- (7) Add to each tube 2 drops of Reagent No. 3.

f. RESULTS

- (1) If a red color develops, the test is positive. This color change may vary from pink to deep red. Compare results with the color standard and record $\pm$ to 5— according to the intensity of color change.
- (2) If no color change occurs, the test is negative.
- (3) Confirm all negative tubes by adding a small amount of powdered zinc. (Dip the tip end of a slightly moist applicator stick into dry zinc dust and shake the dust into the liquid.)
 - (a) Development of a red color following addition of zinc indicates that the negative reading is valid.
 - (b) If no color change occurs upon adding zinc, the reaction may have been positive and the nitrate reduced beyond nitrite. If this occurs, repeat the test to check its validity.

For examples, see Figure 17.

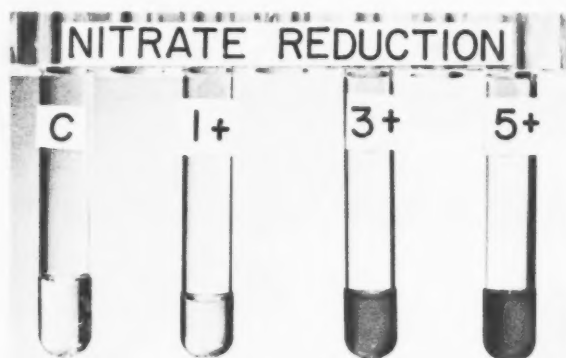


Figure 17 — Nitrate Reduction Test (examples of weak and strong reactions).

Reference:

Virtanen, S. 1960. A study of nitrate reduction by mycobacteria. Acta. Tuberc. Scand. Suppl. 48.

3. Combined Niacin-Nitrate Reduction Test

a. CULTURES

The niacin and nitrate tests may be performed with a single culture slant. The niacin is extracted from the culture with the nitrate-buffer substrate solution, then the nitrate test is performed in the tube containing the culture slant.

b. EQUIPMENT, REAGENTS AND CONTROLS

See niacin test page 53 and nitrate test page 55.

c. PROCEDURE

- (1) To each culture slant, add 2.0 ml of the NaNO_3 substrate solution.
- (2) Place the tubes in a slanted position for 15 minutes.
- (3) Remove 0.5 ml of the substrate and place it in a clean screw-capped test tube to use for the niacin test.
- (4) With the tip of the pipette, dislodge several colonies from the slant into the remaining nitrate substrate in the slant.
- (5) Place these slants in a 37°C water bath for 2 hours.
- (6) Meanwhile, perform the niacin test on the extract set aside in step 3 as follows:
To each tube of extract add 0.5 ml aniline and 0.5 ml of cyanogen bromide. Formation of a yellow color denotes a positive test.
- (7) After the 2 hours at 37°C, remove the slants from the water bath.
- (8) To the liquid in the slant, add the usual nitrate reagents:
 - (a) One drop of reagent No. 1.
 - (b) Two drops of reagent No. 2.
 - (c) Two drops of reagent No. 3.

NOTE: Do not allow reagents to run down the surface of the slant.

f. RESULTS

- (1) Read and record results immediately, comparing the color change with the color standard for intensity of reaction.
- (2) Add zinc dust to negatives to confirm results.

Reference:

Kubica, G. P. 1964. A combined niacin-nitrate reduction test for use in the identification of mycobacteria. *Acta. Tuberc. Scand.* 45:161.

4. Tween 80 Hydrolysis (Degradation) Test

a. CULTURES

Inoculate all slow growers into Tween substrate as soon as sufficient growth appears for transfer.

This test separates the clinically significant scotochromogens and nonphotochromogens from the normally saprophytic representatives of these groups. It also is an excellent confirmatory test for *M. kansasii*.

b. SUBSTRATE

To Prepare

- (1) Combine:
100.00 ml M/15 phosphate buffer pH 7.0
0.5 ml Tween 80.
2.0 ml of a 0.1% aqueous neutral red stock solution.
- (2) Dispense in 2.0-ml amounts into 16 x 125 mm screw-cap tubes.
- (3) Autoclave 10 minutes at 121°C. This should be an amber color liquid after autoclaving.
- (4) Let cool. This substrate may be stored in the dark in the refrigerator for no more than two weeks.

c. CONTROLS

M. kansasii (positive)

Uninoculated tube of substrate (negative).

d. PROCEDURE

- (1) Number tubes of Tween 80 substrate to correspond with culture numbers to be tested.
- (2) Inoculate the substrate with a 3-mm loopful of growth from solid medium (*OR* a loopful of a 7-day-old liquid culture).
- (3) Incubate at 37°C. (Incubate an uninoculated tube of substrate for a negative control.)

e. RESULTS

- (1) Observe tubes for a color change of the liquid from amber to pink or red. *Do not shake tubes for reading.* A positive test occurs when the fluid in the tube, not the cells, turns pink or red.
- (2) Read at 24 hours, record, and discard positives.
- (3) Read at 5 days, record, and discard positives.
- (4) Read at 10-12 days. Record final results and discard all tubes.

For examples, see Figure 18.

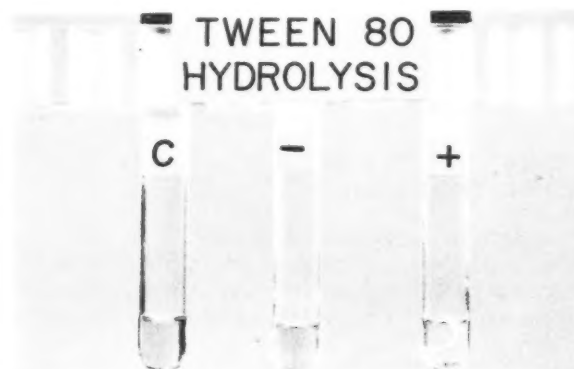


Figure 18 — Tween Hydrolysis Test (examples of different intensity reactions).

Reference:

Wayne, L. G. 1962. Differentiation of mycobacteria by their effect on Tween 80. *Amer. Rev. Resp. Dis.* 86:579.

Wayne, L. G., Doubek, J. R. and Russell, R. L., 1964. Classification and identification of mycobacteria. I. Tests employing Tween 80 as substrate. *Amer. Rev. Resp. Dis.* 90:588.

5. Catalase Tests

a. CULTURES

Cultures may be tested for catalase activity as soon as colony forms are mature. *M. kansasii*, *M. scrofulaceum*, and *M. fortuitum* are the significant cultures with high catalase activity. *M. tuberculosis* resistant to isoniazid may be catalase negative. Perform:

ROOM TEMPERATURE TEST on all cultures.

pH 7/68°C TEST on all nonchromogenic slow growers.

SEMIQUANTITATIVE TEST instead of the room temperature test for more standardized results.

b. EQUIPMENT

Pipettes, 5-ml, 1-ml, or capillary.

Screw-cap test tubes.

Water bath or constant temperature block-heater for pH7/68°C test.

Inoculating spade or loop.

c. REAGENTS

- (1) 30% hydrogen peroxide (Superoxol).

Store in the refrigerator.

- (2) 10% Tween 80 prepared as follows:

Mix 10 ml Tween 80 with 90 ml of distilled water. Autoclave for 10 minutes at 121°C. If Tween settles out during autoclaving, swirl to resuspend. Store in the refrigerator.

- (3) Immediately before performing the test, mix equal parts of Tween solution and Superoxol. (Approximately 0.5 ml of the mixture will be used for one test culture.)

- (4) M/15 phosphate buffer, pH 7.

d. CONTROLS

M. fortuitum (positive).

Uninoculated medium (negative).

Uninoculated pH7 buffer (negative).

e. PROCEDURES

Room Temperature Test

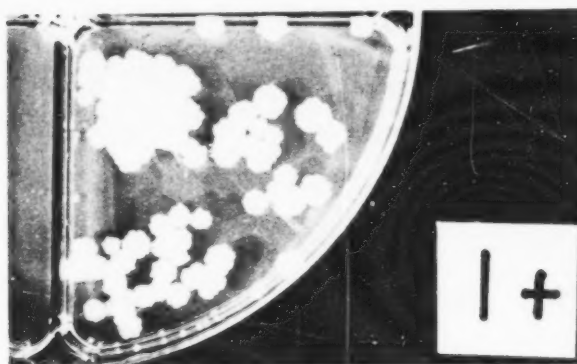
- (1) Add 1-2 drops of the Tween-peroxide mixture to the culture growth on the plate or slant.
- (2) Observe for the formation of bubbles. This may require 5 minutes.

(3) Results

High positive	=	rapid bubbles
Low positive	=	slow bubbles
Negative	=	no bubbles

For examples, see Figures 19, A and B.

Figure 19 — Catalase Activity Observed on 7H-10 Agar Medium



A. A 1+ reaction with bubbling observed near the center of the dish (Upper left portion of picture)



B. Violent "marshmallowy" bubbling (5+)

pH7/68°C Catalase Test

- (1) Number screw-cap tubes to correspond to cultures to be tested. Label tubes for controls.
- (2) With a sterile pipette, add 0.5 ml phosphate buffer pH 7 to each tube.
- (3) With a sterile spade or loop emulsify several colonies from the culture into the buffer.
- (4) Place the tubes containing emulsified colonies in a water bath or constant temperature block-heater at 68°C for 20 minutes.

Time and temperature are important.

- (5) Remove from heat and cool to room temperature.
- (6) Add to each tube 0.5 ml of the Tween-peroxide mixture.
- (7) Observe for formation of bubbles appearing on the surface of the liquid. Hold tubes for 20 minutes before discarding as negative.

(8) Results:

Positive	=	bubbles
Negative	=	no bubbles

R° and pH 7 tests can be performed on the same slant or plate of the culture. Scrape colonies for pH 7 before testing at R°.

References:

Kubica, G. P., et. al., 1966. Differential identification of mycobacteria. I. Tests on catalase activity. Am. Rev. Tuberc. 94:400.

Middlebrook, G. 1954. Isoniazid resistance and catalase activity of tubercle bacilli, a preliminary report. Amer. Rev. Tuberc. 69:471.

Kubica, G. P. and Pool, G. L. 1960. Studies on the catalase activity of acid-fast bacilli. I. An attempt to subgroup these organisms on the basis of their catalase activities at different temperatures and pH. Amer. Rev. Resp. Dis. 81:387.

Semiquantitative Catalase Test

This test may be used instead of the room temperature test for more standardized results, which aid in obtaining a more definite taxonomy.

Preparation of Test Cultures

- (1) Special tubes of medium are prepared by dispensing 5 ml of liquid Lowenstein medium into sterile 18 x 150 mm screw-cap test tubes.

- (2) Inspissate medium with tubes in an upright position to form a butt of solid medium. This may be done in a water bath adjusted to 85°C, if the usual inspissator is not suitable.
- (3) Inoculate surface of butt medium with 0.1 ml or a 3-mm loopful of a 7-day-old liquid culture.
- (4) Incubate at 37°C with caps loose for two weeks.

Procedure

- (1) To a 2-week-old culture, add 1.0 ml of the Tween-peroxide mixture.
- (2) Leave culture in an upright position at room temperature for 5 minutes.
- (3) Measure in millimeters the height of the column of bubbles above the surface of the medium.

Note: A convenient reading guide may be devised by drawing lines at 10-mm intervals across a 3" x 5" card.

(4) Results

Negative = no bubbles

<40 = less than 40 mm bubbles (low)

>50 = more than 50 mm bubbles (high)

For examples, see Figure 20.

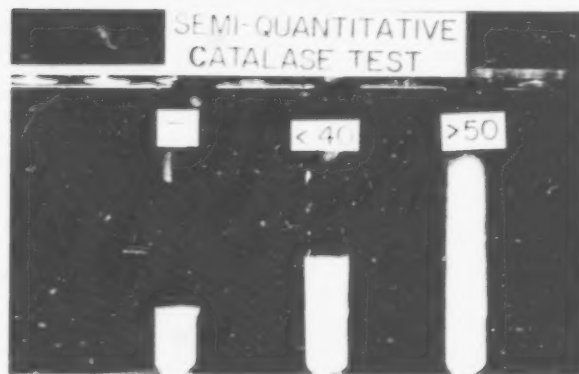


Figure 20 — Semiquantitative Catalase Test

6. Tellurite Reduction

a. CULTURES

Inoculate all slow growing nonchromogenic cultures as soon as sufficient growth appears for transfer.

This test separates *M. avium* - *M. intracellulare* from other slow growers. These are positive at 3 days.

b. MEDIUM

Middlebrook 7H-9 liquid (5 ml in 18 x 150 mm screw-cap tubes).

Label tubes according to culture numbers.

c. REAGENT

0.2% potassium tellurite.

To Prepare:

- (1) Dissolve 0.1 g potassium tellurite in 50 ml distilled water.
- (2) Dispense in several tubes in 2-ml amounts.
- (3) Autoclave 10 minutes.
- (4) Store in the refrigerator.

d. CONTROLS

M. fortuitum or *M. intracellulare* (positive)

Uninoculated broth and reagent (negative)

e. PROCEDURE

- (1) Inoculate the liquid medium with a spadeful of organisms from a culture. Inoculate positive control.
- (2) Incubate at 35-37°C for 7 days. Be certain growth is heavy (turbid).
- (3) With a capillary pipette add 2 drops of the potassium tellurite solution to each tube.
- (4) Reincubate at 35-37°C.
- (5) On the third day following reincubation, read the test results. Do not shake in reading.

f. RESULTS

Positive = black metallic precipitate

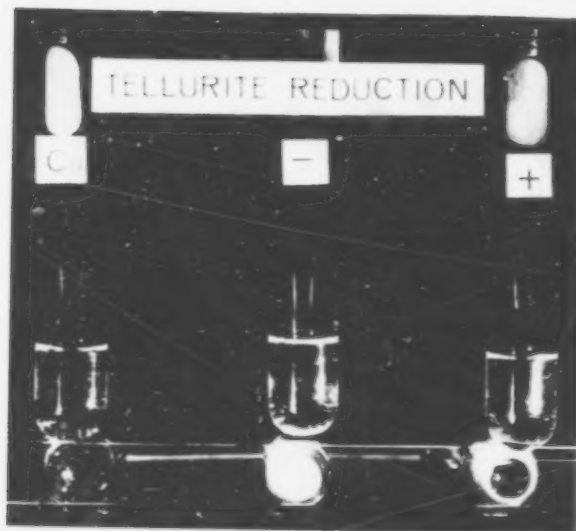
Negative = no black precipitate

For example, see Figure 21.

Reference:

Kilburn, J. O., Silcox, V. A., Kubica, G. P. 1969. Differential identification of mycobacteria V. the tellurite reduction test. Am. Rev. Resp. Dis. 99:94.

Figure 21 — Tellurite Reduction Test



7. Arylsulfatase Test

a. CULTURES

Inoculate all rapid growers to arylsulfatase substrate medium. This test aids in identifying *M. fortuitum*.

Nonchromogenic slow growers may also be inoculated to arylsulfatase to separate the *M. trivale* from the other insignificant nonphotochromogens. (For media, see Jones, et. al.).

This test is also helpful in the identification of *M. marinum*, *M. xenopi*, and *M. flavescens*.

b. MEDIUM

Prepare stock substrate

- (1) Dissolve 2.6 g tri-potassium phenolphthalein disulfate in 50 ml distilled water; this is an 0.08 M solution.
- (2) Sterilize by filtration.
- (3) Store in the refrigerator.

Dubos Broth

Prepare a flask with 200 ml of Dubos broth medium with albumin according to directions on the label.

Dubos Broth with Substrate (arylsulfatase medium)

- (1) Add 2.5 ml of the 0.08 M stock substrate to 200 ml Dubos broth (0.001 M arylsulfatase)
- (2) Dispense aseptically in 2-ml amounts in 16 x 125 mm screw-cap tubes.

c. REAGENT

2 N sodium carbonate (10.6 g anhydrous Na_2CO_3 in 100 ml H_2O).

d. CONTROLS

A tube of uninoculated substrate medium (negative).

A tube of substrate inoculated with *M. fortuitum* (positive).

NOTE: Prepare color standard for reading test results. A standard for reading the arylsulfatase test in Appendix D, p. 137.

e. PROCEDURE

- (1) Label tubes of substrate medium with specimen numbers.
- (2) Inoculate the tubes with 0.1 ml of a 7-day liquid culture of the organism or a spade-ful of organisms from a freshly grown culture.
- (3) Incubate at 37°C. Incubate control substrate medium.
- (4) After 3 days' incubation, remove tubes from the incubator and add not more than 6 drops of 2 N sodium carbonate solution.

f. RESULTS

A color change to pink or red indicates a positive test. Compare results with a set of color standards and record intensity of color (— to 5+).

References:

- Whitehead, J. E. M., Wildy, R. and Engbaek, H. C. 1953. Arylsulfatase activity of mycobacteria. *J. Path. Bact.* 65:451.
- Wayne, L. G., Juarez, W. J. and Nichols, E. G. 1959. Arylsulfatase activity of aerobic Actinomycetales. *J. Bact.* 75:367.
- Kubica, G. P. and Vestal, A. L. 1961. The arylsulfatase activity of acid-fast bacilli. I. Investigation of stock cultures of acid-fast bacilli. *Amer. Rev. Resp. Dis.* 83:728.
- Kubica, G. P. and Beam, R. E. 1961. The arylsulfatase activity of acid-fast bacilli. II. The differentiation of *Mycobacterium avium* from the unclassified Group III non-photochromogenic mycobacteria. *Amer. Rev. Resp. Dis.* 83:733.

Kubica, G. P. and Ridgon, A. L. 1961. The arylsulfatase activity of acid-fast bacilli. III. Preliminary investigation of rapidly growing acid-fast bacilli. Amer. Rev. Resp. Dis. 83:737.

Jones, W. D., Abbot, V. D., Vestal, A. L., Kubica, G. P. 1965. A hitherto undescribed group of nonchromogenic mycobacteria. Am. Rev. Resp. Dis. 94:790.

8. Arylsulfatase Test — Wayne Modification

a. CULTURES

All rapid growers may be inoculated onto Wayne's arylsulfatase medium.

This is a 3-day test employing a commercial agar-base medium.

This substrate medium may also be used as a 2-week test for rapid or slow growers. The procedure is the same except the cultures are incubated for 2 weeks before adding the sodium carbonate.

b. MEDIUM

- (1) Prepare commercial medium as directed on the label.
- (2) After autoclaving, the tubes may be solidified in a slanting or upright position.
- (3) This medium may be stored indefinitely in the refrigerator.

c. REAGENT

2N sodium carbonate.

d. CONTROLS

Uninoculated tube of medium (negative).

Medium inoculated with *M. fortuitum* (positive).

e. PROCEDURE

- (1) Inoculum may be a 7-day-old liquid culture or colonies from an actively growing culture on solid medium.
- (2) Transfer either a 3-mm. loopful or 0.1 ml with a pipette to the test medium.
- (3) Incubate at 37°C.
- (4) After 3 days, add a few drops of 2N sodium carbonate.

f. RESULT

Any color change to red or pink is positive.

(A heavy inoculum produces a more rapid and deeper color change than a light inoculum.)

Reference:

Wayne, L. G. 1961. Recognition of *Mycobacterium fortuitum* by means of a three day phenolphthalein sulfatase test. Tech. Bull. Reg. Med. Tech. 31:123.

9. MacConkey Agar

a. CULTURES

Inoculate all rapid growers onto MacConkey agar. *M. fortuitum* shows growth and sometimes a color change in 5 days.

b. EQUIPMENT

3-mm loop.

Turntable.

c. MEDIUM

MacConkey agar in plates.

d. CONTROLS

An uninoculated plate of medium (negative).

A plate inoculated with *M. fortuitum* (positive).

e. PROCEDURE

(1) Label plates to correspond with the culture numbers.

(2) Place the plate medium-side down on the turntable.

Place a 3-mm. loopful of a 7-day liquid culture in the center of the plate on the surface of the medium. Spin the plate and move the loop slowly along the surface of the medium. This produces inoculum in the form of a helix.

When a turntable is not available, plates may be streaked as for isolated colonies, but the results by this method are not as conclusive.

(3) Incubate plates in a cannister, with the top loose, at 35-37°C without additional CO₂ in the atmosphere.

(4) Read at 5 days and 11 days.

f. RESULTS

No growth = negative

Growth = positive

Growth and color change = positive.

M. fortuitum usually shows growth along the entire length of the helix. With heavy inoculum *M. smegmatis* may show some growth near the beginning of the helix.

Reference:

Jones, W. D. and Kubica, G. P. 1964. The use of MacConkey's agar for the differential typing of *Mycobacterium fortuitum*. Amer. J. Med. Tech. 30:187.

10. Light Exposure

a. CULTURES

The light exposure test should be performed on: (1) all cultures

nonchromogenic after incubation in the dark, and (2) chromogenic cultures that were not incubated continuously in the dark. The culture must be young, actively growing, and have isolated colonies. A change from nonchromogenic to chromogenic is characteristic of the *M. kansasii* photochromogen.

b. MEDIUM

Lowenstein-Jensen (egg medium) slant or 7H-10 plate.

c. INOCULUM

May be:

Diluted sediment of clinical specimen,
Liquid culture (7-10 days) diluted, or
Dilute saline suspension of colonies.

d. CONTROLS

The test procedure provides its control.

e. PROCEDURE

- (1) Inoculate two tubes of egg base or two plates of 7H-10 medium so that isolated colonies will be obtained.
- (2) Shield one of the plates or tubes from light with black or dark paper.
- (3) Incubate in the dark until visible colonies appear on the culture without the shield.
- (4) Remove shield from culture. Record any pigmentation. If nonchromogenic, cover half the tube or plate and expose the other half of the culture to the light of a 30-60 watt lamp at a distance of 8-10 inches for one hour. Loosen the cap of the tube during exposure time.
- (5) Replace shield. With cap still loose, reincubate for 6-12 hours or overnight.
- (6) Compare pigmentation of colonies on exposed half of the culture to that on the unexposed portion.

f. RESULT

A change from nonchromogenic to lemon yellow indicates a photochromogen.

For example, see Figure 10, page 37.

References:

- Runyon, E. H. 1965. Anonymous mycobacteria in pulmonary disease. *Med. Clin. N. Amer.* 43:273.
- Wayne, L. G. and Doubek, F. R. 1964. The role of air in the photochromogenic behaviour of *Mycobacterium kansasii*. *Am. J. Clin. Path.* 42:431.

11. Sodium Chloride Tolerance Test

a. CULTURES

Transfer all positive cultures to 5% sodium chloride medium.

This is a test to confirm rapid growers and the slow growing *M. triviale*. *M. flavescens* sometimes grows on sodium chloride.

b. MEDIUM

5% NaCl in ATS (American Trudeau Society) or Lowenstein-Jensen medium.

ATS or Lowenstein without NaCl as control.

(See page 118 for preparation of medium.)

c. INOCULUM

0.1 ml of a barely turbid suspension of a liquid culture or growth from a slant suspended in sterile water or saline.

d. CONTROLS

The test procedure provides the control.

e. TEST

(1) Inoculate a slant of NaCl test medium and a control slant without NaCl with 0.1 ml inoculum.

(2) Incubate at 35-37°C.

(3) Read once a week for growth.

(4) Cultures may be discarded after 4 weeks' incubation.

f. RESULTS

Positive test = growth on the NaCl and growth on the control.

Negative test = no growth on NaCl but growth on the control.

Reference:

Kestle, D. J., Abbott, V. D., and Kubica, G. P., 1967. Differential identification of mycobacteria. II. Subgroups of Groups II and III (Runyon) with different clinical significance. Am. Rev. Resp. Dis. 95:1041.

12. *M. bovis* Confirmation

Mycobacteria isolated from bovine source and suspected of being *M. bovis* should be confirmed by: (a) inoculating a rabbit and or (b) inoculating 7H-10 medium containing 10 mcg/ml of thiophen-2-carboxylic acid hydrazide (TCH).

M. bovis causes progressive disease in the rabbit and usually does not grow in the presence of TCH.

a. RABBIT INOCULATION

- (1) Transfer several colonies from solid medium to Tween-albumin liquid medium.
- (2) Incubate 7-10 days for turbid growth.
- (3) Dilute liquid culture 1:10 with sterile distilled water or physiological saline.
- (4) Inoculate 1 ml of the 1:10 dilution into the marginal ear vein of a 4-6 lb rabbit.
- (5) Results
 - (a) Rabbits inoculated with *M. bovis* may die of generalized tuberculosis within 90 days. Upon autopsy, lesions in the lungs, spleen and liver indicate *M. bovis*. Make impression smears of these organs and stain for acid-fast bacilli.
 - (b) Rabbits that survive for 90 days are sacrificed. Autopsy revealing only a few small lesions in the lungs and kidney indicate *M. tuberculosis*.
 - (c) If the rabbit dies in less than 6 weeks, autopsy and inoculate another animal if negative for *M. bovis*.

**B. SENSITIVITY TO TCH (THIOPHEN-2-CARBOXYLIC ACID
HYDRAZIDE)**

TCH Medium: Prepare 7H-10 medium containing 10 mcg/ml TCH. This medium may be poured into one section of a drug susceptibility test quadrant plate, or,

Pour TCH medium into each of two sections of a quadrant plate and in the remaining two sections pour control medium without TCH.

- (1) Prepare a 7- to 10-day-old liquid culture as for rabbit inoculum.
- (2) Dilute the culture with sterile saline or distilled water to 1:10 and 1:1000 (10^{-1} and 10^{-3}).
- (3) With a sterile capillary pipette, inoculate one control and one TCH quadrant each with three drops of the 10^{-3} dilution and each of the remaining two quadrants with three drops of the 10^{-1} dilution.
- (4) Incubate 3 weeks at 37°C in 5-10% CO₂ atmosphere.
- (5) Read in the same manner as drug susceptibility test plates comparing growth on TCH with control medium.
- (6) Results

M. bovis does not grow in the presence of TCH at this concentration. However, *M. bovis* strains that are resistant to isoniazid are usually resistant to TCH. Other mycobacteria do grow in the presence of TCH.

References:

- Harrington, R., and Karlson, A. G. 1966. Differentiation between *M. tuberculosis* and *M. bovis* by *in vitro* procedures. Am. J. Vet. Res. 27:1193.
- Vestal, A. L., and Kubica, G. P. 1967. Differential identification of mycobacteria. III. Use of thiacetazone, thiophen-2-carboxylic acid hydrazide and triphenyltetrazolium chloride. Scand. J. Resp. Dis. 48:142.

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G. PROBLEM CULTURES

A culture free of contamination is a necessity in order to obtain reliable results of identification and drug susceptibility tests. In some instances a single specimen may contain more than one species of mycobacteria. Colony characteristics and/or unusual identification and drug test patterns suggest a mixed culture.

1. Contaminated

a. MATERIALS AND EQUIPMENT FOR DECONTAMINATION

Sterile screw-cap test tubes
Sterile capillary pipettes
Wire loop or straight needle
Sterile distilled water
4% NaOH, sterile
2N HCl, sterile
Phenol red or other indicator
Microscope slides and stains
Lowenstein-Jensen and/or 7H-10 media

b. PROCEDURE FOR DECONTAMINATION

- (1) Number screw-cap tubes and slides for all cultures to be decontaminated.
- (2) With a capillary pipette, place 4 drops of sterile distilled water in each tube.
- (3) Pick a colony from the culture with a wire loop or spade. Suspend the organisms in the water by slowly grinding them against the side of the tube.
- (4) Transfer a drop of this solution to a slide and stain for acid-fastness.
- (5) Add 4 drops of 4% NaOH to the culture suspension in the tube.
- (6) Tighten cap of tube and shake gently by hand to mix.
- (7) Let stand at room temperature for 10 minutes. Shake occasionally. *Do not incubate at 37°C.*
- (8) Neutralize with 2N HCl using a drop of indicator such as phenol red.
- (9) Add 2 to 3 ml sterile distilled water.
- (10) Inoculate one tube of L-J medium with 0.1 ml of this suspension and one tube with a 1:10 dilution.
- (11) With a wire loop, streak a plate of 7H-10 with a loopful of the undiluted suspension.
- (12) When only one type of medium is available:
 - (a) L-J: inoculate 2 additional tubes with the 10^{-2} and 10^{-4} dilutions.

- (b) 7H-10: streak an additional plate with the 10^{-1} dilution.
- (13) Incubate subcultures. Read at 24 hours, one week, and each week for 6 weeks.
 - (14) When growth appears, select isolated colonies for culture identification procedure. Make smears to determine acid-fastness and purity.
 - (15) When necessary to repeat the decontamination process, it is sometimes advisable to reverse the procedure. To do this, add the 2N HCl to the suspension of bacilli (at step 5) and in step 8 neutralize with the 4% NaOH.

When more than one colony form is seen on a culture, make a separate suspension with each. Label the subculture tubes with the specimen number and type of colony selected. Inoculate separate plates and tubes of medium with each.

2. Mixed

Suspect a mixed culture when:

More than one colony form is present.

Identification test pattern does not conform to that of any species.

Unusual drug susceptibility test results occur, for example, two or more colony forms appear on the control medium with one colony form on drug medium.

a. MATERIALS AND EQUIPMENT

Plates and tubes of medium.

Loop or straight wire for picking colonies.

Microscope slides and stains.

b. PROCEDURE FOR SEPARATION

- (1) Label media and slides with culture numbers and the colony forms selected.
- (2) Keep accurate records of all colony separations. Label media with culture numbers followed by letters (a, b, etc.), and keep colony descriptions in record book.
- (3) Pick each colony form to separate media and streak for isolated colonies. If the culture has confluent growth after incubation, it is difficult to determine separate colony forms.
- (4) Make a smear and stain for acid fastness.
- (5) Incubate cultures and read at 24 hours, 1 week, and each week for 6 weeks.

- (6) Examine growth for pure colony forms and compare with records of original colony descriptions.
- (7) One colony form denotes a pure culture. However, cultures of mycobacteria other than *M. tuberculosis* and *M. bovis* frequently have more than one type of colony within a culture. Smooth colonies may be domed and spreading with an occasional rough variant. The number of rough variants in a culture usually increases on repeated culture transfer.
- (8) When a pure acid-fast colony form is isolated, transfer to medium and perform the necessary identification tests.
- (9) An astute bacteriologist should be able to separate mixed strains when they occur.

SECTION III. DRUG SUSCEPTIBILITY TESTS

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A. INTRODUCTION TO SECTION III

Drug susceptibility testing is one of the most difficult procedures to standardize for the diagnostic mycobacteriology laboratory. Briefly, some of the reasons for these difficulties are:

1. Stability of drugs under different methods of sterilization and storage. (See Table III.)
2. Alteration in the antimycobacterial activity of various drugs when incorporated into different media. (See Table IV.)
3. The use of at least three methods for determining susceptibility.

For a more detailed discussion of these reasons, see Kubica and Dye, Laboratory Methods for Clinical and Public Health: Mycobacteriology, Chapter VII, "Drug Susceptibility Testing."

The procedure now recommended by the NCDC and in general use by the Veteran's Administration and Armed Forces is an economical version of the proportion method of Canetti et al. In the proportion method, the laboratory must determine that proportion of the bacterial population which is resistant to the concentration of drugs used for testing. When 1% or more of the organisms which grow on the control are observed to grow on a particular drug, then, for clinical purposes, that particular drug is not, or soon will not be, valuable for the treatment of a given patient.

The drug susceptibility test may be performed by either the direct or the indirect method.

The direct drug susceptibility test is performed on a specimen in which acid-fast bacilli can be demonstrated on a smear of the digested, concentrated specimen. The inoculum for the test is the specimen concentrate diluted according to the number of acid-fast bacilli seen on the smear.

The indirect drug susceptibility test is performed by using a subculture from a primary culture as the inoculum. Although the direct test is preferred, the indirect test may be performed when one of the following situations occurs:

The smear was negative and the culture has growth.

The growth on the control of the direct test is inadequate for a reliable report.

A reference culture is sent in by another laboratory.

Table III

Percent of Active Drug Remaining after Autoclaving, Filtering, and Freezing.

Drug at 10,000mcg./ml.	Method of sterilization				Stored at -20°C.		
	Autoclave	Seitz	Glass	Millipore	3 mo.	5 mo.	6 mo.
Isoniazid	90	92	100	98	98	98	97
Streptomycin	90	88	95	95	95	92	90
PAS	100	90	100	100	85	80	75
Kanamycin	98	90	96	98	98	98	98
Ethionamide	90	80	90	90	95	88	85
Viomycin	94	95	98	95	98	95	94
Ethambutol	98	80	88	90	90	90	90
Cycloserine	0	98	98	98	85	75	65
Pyrazinamide	100	100	100	100	100	100	100
Rifampin	not tested			100	91	86	

Table IV

Range of Minimal Inhibitory Concentrations of Drugs, in 7H-10 and Lowenstein Medium, Tested with 20 Wild Strains of *M. tuberculosis*.

Drug	Medium	
	7H-10 mcg/ml	Lowenstein mcg/ml
Isoniazid	0.05- 1.5	0.05- 1.5
Streptomycin	1.0 - 2.0	1.0 - 5.0
PAS*	1.0 - 2.0	0.25- 0.5
Kanamycin	1.0 - 2.5	8.0 -12.0
Ethionamide	1.5 - 4.0	6.0 -15.0
Viomycin	2.0 - 5.0	15.0 -18.0
Ethambutol	2.0 - 6.0	2.0 - 6.0
Cycloserine*	30	20
Pyrazinamide	18-22	18-22
Rifampin	0.05- 0.2	0.5 - 2.0

* PAS and cycloserine seem to have lower minimal inhibitory concentrations in Lowenstein than in 7H-10 medium. Because of the bacteriostatic effect of these two drugs, minute colonies remain undetected on egg medium but they will be detected on 7H-10 medium.

For performing the tests, inoculate medium containing concentrations of the primary drugs and control medium with no drug. In the 7H-10 medium these are:

Isoniazid (INH)	0.2 mcg/ml
Streptomycin (SM)	2.0 mcg/ml
Para-aminosalicylic acid (PAS)	2.0 mcg/ml
Control	no drug

When the mycobacteria show 1% or more resistance to these concentrations of drugs, inoculate media containing higher concentrations and/or other drugs. See Table V.

To determine the proportion of resistance, the number of colonies on the control and the number of colonies on the drug medium must be determined. If the growth is confluent (4+), it cannot be counted. Two dilutions of inocula, usually one-hundredfold apart are planted onto duplicate plates of medium. This should give countable colonies on at least one of the controls. (See examples, Section C, Reporting Drug Susceptibility Tests.)

A test is considered valid when there are 50 or more colonies on at least one of the controls.

For preparation of media, see page 88.

Table V
Concentration of Drug in Susceptibility Test Medium (7H-10)

Drug	mcg /ml
Isoniazid	0.2, 1.0, and 5.0
Streptomycin	2.0 and 10.0
PAS	2.0 and 10.0
Kanamycin	5.0
Ethionamide	5.0
Viomycin	5.0
Ethambutol	5.0
Cycloserine	50.0
Pyrazinamide (pH 5.5)*	50.0
Rifampin	1.0

* To adjust 7H-10 medium to the required acidity, pH 5.5, it is necessary to alter the ratio of the buffering phosphate salts. (Stattmeier, 1967)

B. DIRECT TEST

Materials and Equipment

Medium: 7H-10 in quadrant Petri plates.

2 plates per specimen, one for each dilution of inoculum.

Sterile saline, 4.5-ml volumes in screw-cap tubes.

Pipettes, Pasteur capillary and 1-ml.

Procedure

1. Stain and read smears of concentrated clinical specimens, recording the average number of acid-fast bacilli found per oil immersion field. Example: After finding a field with acid-fast bacilli, read at least 20 additional fields and take an average. The bacilli in a sputum concentrate are often found in clumps.
2. Select dilutions of concentrate for inocula on the basis of positive smear results as follows:

Microscopy	Inoculate
Less than 1 AFB /field	Undilute and 10^{-2} (1:100)
1-10 AFB/field	10^{-1} and 10^{-3} (1:10 and 1:1000)
More than 10 AFB /field	10^{-2} and 10^{-4} (1:100 and 1:10,000)

3. Dilutions may be made with 0.5 ml of the albumin concentrate and 4.5 ml of sterile water or saline. Mix and transfer successively 0.5 ml to 4.5 ml for the required dilutions.
4. With a capillary pipette inoculate three drops onto each quadrant of the drug and control media. Inoculate one plate with the highest selected dilution and duplicate plate with the lower dilution.
For patients who have been under treatment, it is advisable to inoculate one plate with undiluted inoculum. Organisms from such individuals may be stained, but they may be non-cultivable because of the action of the drugs.
5. Place plates, medium side down, in individual polyethylene plastic bags and seal.
6. Incubate in an atmosphere of 5-10% carbon dioxide at 35-37°C.
7. Plates in individual bags may be read weekly, but most strains of *M. tuberculosis* will not produce visible colonies until the end of the second or third week.
8. Cultures should not be discarded as negative until the end of the fifth week. Some few strains, especially those resistant to drugs, may require 4 to 5 weeks for microscopic colonies to appear.
9. Examine plates with the aid of a dissecting microscope using

30-60X total magnification and transmitted light. Sometimes colonies are visible macroscopically on the control but are only visible microscopically on the drug containing medium. Do not discard a plate as negative without reading it with the aid of magnification.

10. Record bacterial growth on the control and drug medium as follows:

Confluent (500 or more colonies)	++++	(4+)
Almost confluent (200-500 colonies)	+++	(3+)
100-200 colonies	++	(2+)
50-100 colonies	+	(1+)
Less than 50		Actual Count

11. Record colony characteristics on the control medium.
12. Report results obtained with both dilutions and calculate percent resistance. (See reporting, page 82.)

C. INDIRECT TEST

Whenever possible, the direct drug susceptibility test procedure should be used. Under circumstances requiring the indirect drug susceptibility test, growth from a culture of mycobacteria on drug free liquid or solid medium is used as inoculum. For the test to be valid the inoculum must be large enough for good growth (50-100 colonies on the control quadrant) but not large enough to simulate drug resistance due to the growth of spontaneously occurring drug resistant mutants.

TO PREPARE INOCULUM FROM GROWTH ON SOLID MEDIUM:

1. Remove approximately 2-5 mg of growth from drug free medium. Pick a small portion of each colony, if possible.
2. Transfer to a sterile 16 x 125 mm screw-cap tube containing 6-8 glass or plastic beads* and 3 ml of Tween-albumin medium.
3. Homogenize the cells on a test tube mixer for 5-10 minutes.
4. Allow the large particles to settle. Withdraw the supernate and adjust the density to approximately MacFarland No. 1 standard with sterile distilled water or 0.85% saline.**
5. Dilute to 10^{-2} and 10^{-4} in sterile distilled water or 0.85% saline.
6. Proceed as for direct test for inoculation, incubation, reading, and reporting. Be sure to indicate "indirect test" in report.

* Glass beads 1-2 mm in diameter or high heat polystyrene plastic beads such as Styron® 700, 27 clear, No. 7, from Dow Chemical Co.
 ** Prepare MacFarland No. 1 Standard by adding 0.1 ml of 1% BaCl_2 to 9.9 ml of 1% H_2SO_4 .

TO PREPARE INOCULUM FROM CULTURES HAVING INSUFFICIENT GROWTH FOR MACFARLAND NO. 1 STANDARD:

1. Transfer a part of each colony to Tween-albumin medium, such as Dubos or 7H-9.
2. Incubate for 7 days or until as turbid as the MacFarland No. 1 standard.
3. Dilute to 10^{-3} and 10^{-5} in sterile water or saline.
4. Inoculate the two dilutions, incubate, read, and report as described for the direct test.

When strains of mycobacteria show more than 1% resistance to the low concentrations of the primary drugs, the strain should be inoculated onto medium containing higher concentrations.

These are:

INH: 1.0 mcg/ml and 5.0 mcg /ml
SM : 10.0 mcg/ml
PAS: 10.0 mcg/ml

If susceptibility tests are requested against the secondary drugs, see page 89 for concentrations.

D. REPORTING THE DRUG SUSCEPTIBILITY TEST

1. The report should contain:
The type of test, DIRECT or INDIRECT.
The amount of growth on the control medium.
The amount of growth on the drug medium.
The concentration of drug in the medium.
A rough calculation of the percent of the bacilli resistant to the drug.
2. This formula may be used to calculate percent:

$$\frac{\text{Number of colonies on the drug}}{\text{Number of colonies on the control}} \times 100 = \% \text{ resistance}$$

Example:
Test a.

	Growth		% Resistant
	undilute	10 ⁻²	
Control	4+	150	
INH 0.2 mcg/ml	1+	9	6%
SM 2.0 mcg/ml	0	0	0%
PAS 2.0 mcg/ml	0	0	0%

$$\frac{9 \text{ colonies}}{150 \text{ colonies}} \times 100 = 6\%$$

In this test 6% of the organisms are resistant to 0.2 mcg/ml INH.

Test b.

	Growth		% Resistant
	undilute	10 ⁻²	
Control	4+	60	
INH 0.02 mcg/ml	120c	0	2%
SM 2.0 mcg/ml	0	0	0%
PAS 2.0 mcg/ml	0	0	0%

The undilute inoculum in Test b gave 4+, or colonies too numerous to count. The hundredfold dilution gave 60 countable colonies. Therefore 4+ = 60 × 100, or 6000 colonies. Using this figure the proportion of resistant organisms may be calculated as 2% resistant to 0.2 mcg/ml INH.

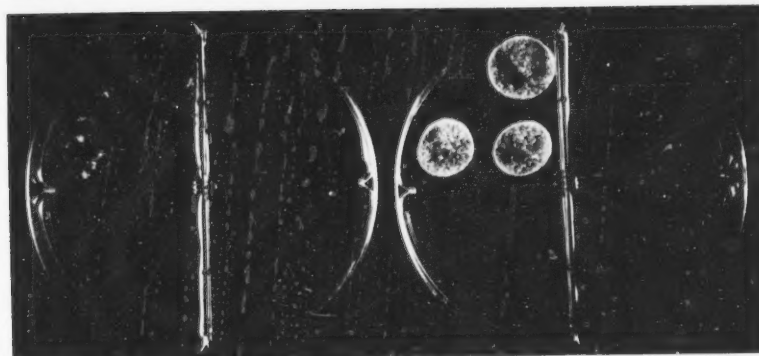
$$\frac{120}{6000} \times 100 = 2\%$$

For other examples, see Figures 22-25.

Figure 22

CON.

PAS 2.0



INH 0.2

SM 2.0

The organisms inoculated onto these plates were susceptible to 0.2 mcg/ml INH (isoniazid), 2.0 mcg/ml SM (streptomycin), and 2.0 mcg/ml PAS (para-amino-salicylic acid).

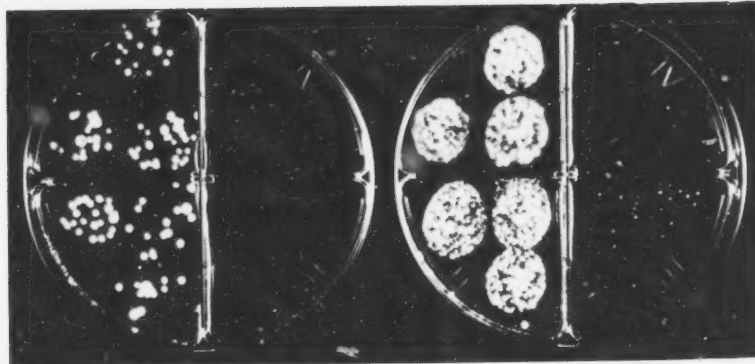
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Laboratory Report EXAMINED MICROSCOPICALLY BY: ☒ ZIEHL NEELSEN ☐ FLUOROCHROME ACID FAST BACILLI ☒ Found ☐ Not Found ☐ Rare ☐ Few ☒ Numerous FUNGI OR YEAST ☐ Found ☐ Not Found ☐ Spill Given To Mycology Lab		DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR ☒ DIRECT ☐ INDIRECT		IDENTIFICATION ☒ <i>M. tuberculosis</i> ☐ <i>M. bovis</i> ☐ <i>M. kansasii</i> ☐ RUNYON GROUP II SCOTCHROMOGEN ☐ <i>M. scrofulaceum</i> ☐ Tap water ☐ RUNYON GROUP III NON PHOTOCROMOGEN ☐ <i>M. intracellulare</i> ☐ <i>M. fortis</i> ☐ <i>M. gastri</i> ☐ <i>M. triviale</i> ☐ RUNYON GROUP IV RAPID GROWERS ☐ <i>M. fortuitum</i> ☐ <i>M. flavescens</i> ☐ Other																																																						
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SAMPLE LABORATORY REPORT FORM

Figure 23

CON.

PAS 2.0



INH 0.2

SM 2.0

The inoculum (10^1) on the right shows 4+ growth on the controls and INH quadrants, 23 colonies on SM, and 2 on PAS. A 100-fold dilution (10^3) of the inoculum on a second drug plate shows 59 colonies on the control, 54 on INH, and none on SM and PAS. Hence, 54/59 gives 92% resistance to INH. To determine percent resistant to SM and PAS, multiply $59 \times 100 = 5900$ colony units inoculated on to each quadrant of the 10^1 plate.

$$23/5900 \times 100 = 0.4\% \text{ R to SM.}$$

$$2/5900 \times 100 = 0.03\% \text{ R to PAS.}$$

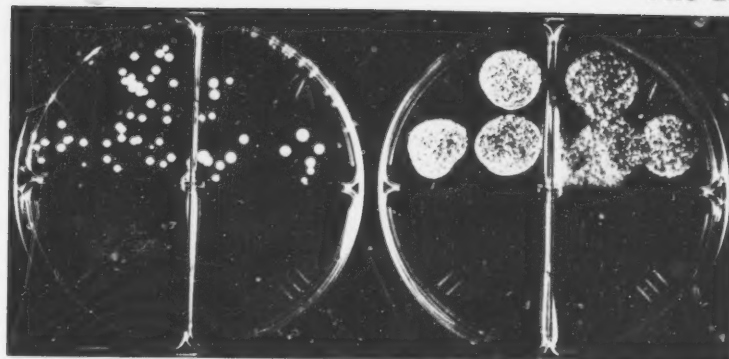
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IDENTIFICATION ☐ <i>M. tuberculosis</i> ☐ <i>M. bovis</i> ☒ <i>M. kansasii</i> RUNYON GROUP II SCOTOCHROMOGEN ☐ <i>M. scrofulaceum</i> ☐ Tap water RUNYON GROUP III NON PHOTOCROMOGEN ☐ <i>M. intracellulare</i> ☐ <i>M. terrae</i> ☐ <i>M. goodii</i> ☐ <i>M. triviale</i> RUNYON GROUP IV RAPID GROWERS ☐ <i>M. fortuitum</i> ☐ <i>M. flavesens</i> ☐ Other		Date Received and Laboratory Specimen Number Month Day Year Form No. Specimen Number																																									
Date Reported 1st 2nd 3rd ☒		Form Control Number																																									

SAMPLE LABORATORY REPORT FORM

Figure 24

CON.

PAS 2.0



INH 0.2

SM 2.0

The inoculum on the plate at the right (10^{-1}) is too heavy to determine actual colony counts. In the hundredfold dilution of inoculum (10^{-3}) on the plate to the left we count 41 colonies on the control and 17 on the PAS quadrant. Hence, the apparent resistance to PAS observed in the heavily inoculated plate is a true one, and approximately 41% of the population was resistant to PAS ($17/41 \times 100 = 41\%$).

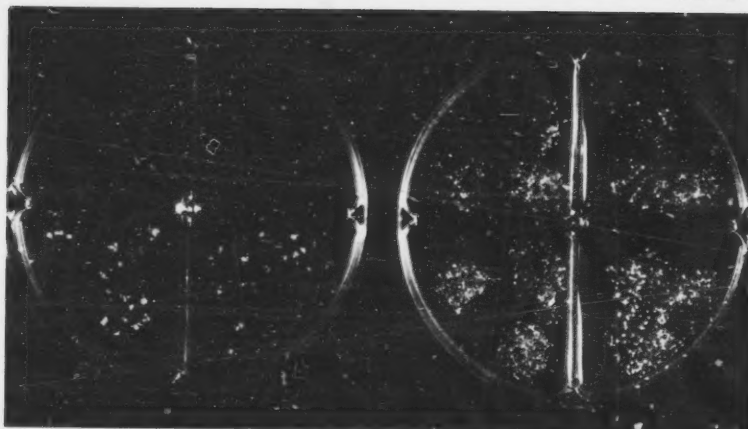
PLEASE PRINT CAREFULLY		State		City		Reprint	
Laboratory Report EXAMINED MICROSCOPICALLY BY ☒ ZIEHL-NEESEN ☐ FLUOROCHROME ACID FAST BACILLI Present Not Present ☒ Rare ☒ Few ☐ Numerous FUNGUS OR YEAST Present Not Present Spore Green T. Microscopic Lab.		DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR ☒ DIRECT ☐ INDIRECT				IDENTIFICATION ☐ <i>M. tuberculosis</i> ☐ <i>M. bovis</i> ☐ <i>M. kansasii</i> RUNYON GROUP II SCOTOCHROMOGEN ☒ <i>M. scrofulaceum</i> Tap water RUNYON GROUP III NON PHOTODROMOGEN ☐ <i>M. intracellulare</i> ☐ <i>M. terrae</i> ☐ <i>M. gastri</i> ☐ <i>M. triviale</i> RUNYON GROUP IV RAPID GROWERS ☐ <i>M. fortuitum</i> ☐ <i>M. flavescens</i> ☐ Other	
		Drug (g/ml)	Growth* at Dils	Percent of Pop			
			10 ⁻¹	10 ⁻³			
		CONTROL 0.0	4+	41%			
		INH 0.2	0	0%			
		INH 1.0	0	0%			
		SM 2.0	0	0%			
		SM 10.0	0	0%			
		PAS 2.0	3+	17%	41%		
		PAS 10.0					
		KM 5.0					
		VM 5.0					
		ETA 5.0					
		CS 20.0					
		ETH 4.0					
SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE ☐ Insufficient Amount ☐ Leaked in Transit ☐ Improper Container ☐ Contaminated		* 4+ = 500-1000 3+ = 200-500 2+ = 100-200 1+ = 50-100 0 = 10C = 10 colonies				TENTATIVE IDENTIFICATION 50 recanted & followed by 10C = 10 colonies	
Date Received and Laboratory Specimen Number Month Day Year Form No. Specimen Number		Date Reported 1st 2nd 3rd ☒				Form Control Number	

SAMPLE LABORATORY REPORT FORM

Figure 25

PAS 2.0

SM 2.0



CON.

INH 0.2

There are countable colonies on all quadrants, even the more heavily inoculated plate. To actually determine the percentage resistance, there are 48 colonies on the control, 32 on INH, 49 on SM, and 37 on PAS. The percentage resistance is 67 for INH, 100 for SM, and 77 for PAS.

$$37/48 \times 100 = 77\% \text{ R to PAS}$$

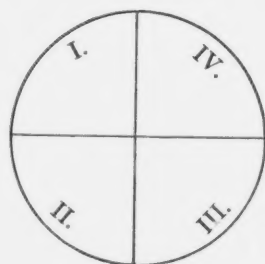
$$32/48 \times 100 = 67\% \text{ to INH}$$

$$49/48 \times 100 = 100\% \text{ R to SM}$$

PLEASE PRINT CAREFULLY		State (Zip Code)		Repeat																																																						
Laboratory Report EXAMINED MICROSCOPICALLY BY: ☒ ZIEHL NEELSEN ☒ FLUOROCHROME ACID FAST BACILLI Found Not Found ☒ Rare ☐ Few ☐ Numerous FUNGUS OR YEAST Found Not Found Split Given To: Microbiology Lab		DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR ☒ DIRECT ☐ INDIRECT		IDENTIFICATION ☐ <i>M. tuberculosis</i> ☐ <i>M. bovis</i> ☐ <i>M. kansasii</i> RUNYON GROUP II SCOTOCHROMOGEN ☐ <i>M. scrofulaceum</i> Tap water RUNYON GROUP III NON PHOTOCROMOGEN ☒ <i>M. intracellulare</i> ☐ <i>M. terrae</i> ☐ <i>M. goodii</i> ☐ <i>M. triviale</i> RUNYON GROUP IV RAPID GROWERS ☐ <i>M. fortuitum</i> ☐ <i>M. flavescens</i> ☐ Other																																																						
		<table border="1"> <thead> <tr> <th>Drug (µg/ml)</th> <th>Growth* at Dils</th> <th>Percent of Pop</th> </tr> <tr> <th></th> <th>10⁰</th> <th>10⁻²</th> </tr> </thead> <tbody> <tr> <td>CONTROL 0.0</td> <td>3+</td> <td>48C</td> <td>100</td> </tr> <tr> <td>INH 0.2</td> <td>2+</td> <td>32C</td> <td>67%</td> </tr> <tr> <td>INH 1.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>SM 2.0</td> <td>3+</td> <td>49C</td> <td>100%</td> </tr> <tr> <td>SM 10.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>PAS 2.0</td> <td>2+</td> <td>37C</td> <td>77%</td> </tr> <tr> <td>PAS 10.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>KM 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>VM 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>ETA 5.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>CS 20.0</td> <td></td> <td></td> <td></td> </tr> <tr> <td>ETH 2.0</td> <td></td> <td></td> <td></td> </tr> </tbody> </table>	Drug (µg/ml)	Growth* at Dils	Percent of Pop		10 ⁰	10 ⁻²	CONTROL 0.0	3+	48C	100	INH 0.2	2+	32C	67%	INH 1.0				SM 2.0	3+	49C	100%	SM 10.0				PAS 2.0	2+	37C	77%	PAS 10.0				KM 5.0				VM 5.0				ETA 5.0				CS 20.0				ETH 2.0					
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SAMPLE LABORATORY REPORT FORM

E. PREPARATION OF MEDIUM



Primary Drug Plates

Quadrant I.	Control.
Quadrant II.	INH 0.2 mcg/ml.
Quadrant III.	SM 2.0 mcg/ml.
Quadrant IV.	PAS 2.0 mcg/ml.

For one set of quadrant plates (see Table VI):

Prepare four flasks of 7H-10 medium base with 180 ml each.

Cool to 50-52°C.

Four tubes of OADC, 20 ml each, warmed to room temperature.

Sterile solutions of drug (see Section G).

TABLE VI
Preparation of Primary Drug Medium

Drug Medium Concentration		To Volume Of Medium	Add	Of Stock Drug
Control		200 ml	0	0
INH	0.2 mcg /ml	200 ml	0.4 ml	100 mcg /ml
SM	2.0 mcg /ml	200 ml	0.4 ml	1000 mcg /ml
PAS	2.0 mcg /ml	200 ml	0.4 ml	1000 mcg /ml

1. To flask No. 1, add 20 ml sterile OADC enrichment. Mix well. Immediately dispense 5-6 ml of medium into quadrant I of each plate. (Control)
2. To flask No. 2, add 20 ml sterile OADC and 0.4 ml of sterile 100 mcg/ml solution of INH. Mix and immediately dispense 5-6 ml into quadrant II of each plate. (0.2 mcg/ml)
3. To flask No. 3, add 20 ml sterile OADC and 0.4 ml of sterile 1000 mcg/ml solution of SM. Mix well and immediately dispense 5-6 ml into quadrant III of each plate. (2.0 mcg/ml)
4. To flask No. 4, add 20 ml sterile OADC and 0.4 ml of sterile 1000 mcg/ml solution of PAS. Mix well and dispense 5-6 ml into quadrant IV of each plate. (2.0 mcg/ml)

For higher concentrations of primary drugs and concentrations of other anti-tuberculosis drugs, see Table VII:

Table VII
Preparation of Other Anti-tuberculosis Drug Media

Drug	Medium* Concentration	To Volume Of Medium	Add	Of Stock Drug
Isoniazid	1.0 mcg/ml	200 ml	0.2 ml	1000 mcg /ml
Isoniazid	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg /ml
Streptomycin	10.0 mcg/ml	200 ml	2.0 ml	1000 mcg /ml
PAS	10.0 mcg/ml	200 ml	2.0 ml	1000 mcg /ml
Kanamycin	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg /ml
Ethionamide	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg /ml
Viomycin	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg /ml
Ethambutol	5.0 mcg/ml	200 ml	1.0 ml	1000 mcg /ml
Cycloserine	50.0 mcg/ml	200 ml	1.0 ml	10,000 mcg /ml
Rifampin	1.0 mcg/ml	200 ml	0.2 ml	1000 mcg /ml

* Each plate must have a control quadrant without drug for accurate reading of test results.

F. STORAGE OF DRUG MEDIUM

All media should be stored in the dark in sterile containers. Medium containing the following drugs may be stored at room temperature for 2 weeks without appreciable loss of drug potency:

Isoniazid
Streptomycin
PAS
Kanamycin
Viomycin
Ethionamide
Ethambutol
Rifampin

In the refrigerator (4-10°C) these drugs remain stable in 7H-10 medium for as long as 2 months, but because stored media occasionally dehydrate, drug plates should be used within 1 month of preparation. Other problems, such as lack of refrigerator space and contamination may be encountered, making it impractical to prepare a 2-month supply.

Media containing cycloserine or pyrazinamide should not be stored at room temperature. Such media remain stable when stored at 4-10°C for as long as one month.

G. PREPARATION OF DRUGS FOR SUSCEPTIBILITY TEST MEDIA

1. Drug Potencies

The true potency of the drug is the number of micrograms of active drug per milligram total weight of the product. Not all antimicrobial drugs have been isolated in pure form and a portion of their weight may be due to impurities or to the sulfate or other radical component of the molecule. The potency should be stated on the vial; otherwise it is necessary to consult the manufacturer for this information. Each lot of partially purified drugs will vary from previous ones, and the potency of one lot may not be the same as that of another lot.

2. Stock Solutions

Prepare stock solution and media the same day.

- a. Drugs may be obtained in the sterile lyophilized form. Instructions accompanying the vial state the potency of the final solution when prepared according to directions. This sterile lyophilized drug reconstituted with sterile distilled water by aseptic technique requires no further sterilization. To obtain working stock solutions of the required concentration, dilute with sterile distilled water. Use a separate sterile pipette for each dilution. Solutions should be mixed well and all measurements made accurately.
- b. Bulk drug in powder form that must be weighed-out from a stock vial and dissolved in distilled water requires sterilization. The membrane filter, such as Millipore, is recommended as a uniform method of sterilization. Some of the drugs lose potency when autoclaved or filtered through Seitz or sintered glass.

Two Millipore membrane filter models are available:

- (a) Micro - syringe filter holder to attach to a 10-ml syringe having Luer fittings.
- (b) Micro-analysis filter holder plus a 125-ml filtering flask.

The micro - syringe requires no vacuum source and is easily cleaned by rinsing in tap water, soaking overnight in distilled water, and drying in the air.

The 25 mm HA filter, porosity 0.45 μ , fits either of the micro models. Prefilter type AP20 is placed on top of the filter paper.

1. When sterilizing, assemble the filter holder with prefilter and filter paper in place.
2. Autoclave 10-15 minutes at 120-121°C.

3. Allow autoclave pressure to rise slowly.
4. At the end of the sterilization period, allow the autoclave to return slowly to normal. Sudden pressure changes may damage the delicate membrane of the filter.
5. Let filter cool to room temperature before filtering the drugs. Filtering volumes less than 10 ml results in unnecessarily large loss of potency due to the drug being absorbed during the early part of the filtration process.

To calculate the weight of drug necessary to prepare 10 ml of a 10,000 mcg/ml solution, this formula may be used:

$$\frac{10,000}{\text{Potency}} \times 10 = \text{milligrams to weigh}$$

Example: Potency of dihydro-streptomycin sulfate =
800 mg of active SM per 1000 mg

$$\frac{10,000}{800} \times 10 = 125$$

Dissolve 125 mg in 10 ml water for 10,000 mcg SM per ml.

Preparation of Isoniazid (INH) Solution

(10 mg isoniazid = 10 mg INH)

1. Dissolve 100 mg (100,000 mcg) isoniazid in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 1 ml of 1000 mcg/ml to 9 ml sterile water. This is a 100 mcg/ml solution.
5. Add 0.4 ml of 100 mcg/ml to 200 ml medium to obtain a final concentration of 0.2 mcg INH per ml of medium.

Preparation of Streptomycin (SM) Solution

(12.5 mg dihydro streptomycin sulfate = 10 mg SM)

1. Dissolve 125 mg of dihydro streptomycin sulfate in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 0.4 ml of 1000 mcg/ml solution to 200 ml of medium to obtain a final concentration of 2.0 mcg SM per ml of medium.

Preparation of p-aminosalicylic acid (PAS) Solution
(5.7 gm sodium p-aminosalicylate = 5 gm PAS)

1. Dissolve 114 mg sodium p-aminosalicylate in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 0.4 ml of 1000 mcg/ml to 200 ml medium to obtain a final concentration of 2.0 mcg PAS per ml medium.

Preparation of Kanamycin (KM) Solution
(1000 mg kanamycin sulfate = 832 mg KM)

1. Dissolve 121.5 mg kanamycin sulfate in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 1 ml of 1000 mcg/ml to 200 ml medium to obtain a final concentration of 5 mcg KM per ml medium.

Preparation of Ethionamide (ETA) Solution
(610 mg ethionamide hydrochloride = 500 mg ETA)

1. Dissolve 122 mg of ethionamide hydrochloride in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 1 ml of 1000 mcg/ml to 200 ml of medium to obtain a final concentration of 5 mcg ETA per ml medium.

Preparation of Viomycin (VM) Solution
(12.2 mg viomycin sulfate = 10 mg VM)

1. Dissolve 122 mg of viomycin sulfate in 10 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 1 ml of 1000 mcg/ml to 200 ml of medium to obtain a final concentration of 5 mcg VM per ml of medium.

Preparation of Ethambutol (EMB) Solution

(10 mg d-ethambutol = 10 mg EMB)

1. Dissolve 100 mg d-ethambutol in 10 ml distilled water.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 1 ml of 1000 mcg/ml to 200 ml of medium to obtain a final concentration of 5 mcg EMB per ml of medium.

Preparation of Cycloserine (CS) Solution

(10 mg cycloserine = 10 mg CS)

1. Dissolve 100 mg cycloserine in 10 ml distilled water. This gives 10,000 mcg/ml.
2. Sterilize by Millipore filter.
3. Add 1 ml of 10,000 mcg/ml to 200 ml of medium to obtain a final concentration of 50 mcg CS per ml of medium.

Preparation of Rifampicin (Rifampin) Solution

(10 mg rifampin = 10 mg rifampin)

1. Dissolve 100 mg rifampin in 10.0 ml distilled water. This is a 10,000 mcg/ml solution.
2. Sterilize by Millipore filter.
3. Add 1 ml to 9 ml sterile distilled water. This is a 1000 mcg/ml solution.
4. Add 0.2 ml of 1000 mcg/ml to 200 ml medium to obtain a final concentration of 1.0 mcg rifampin per ml of medium.

For storage of drugs, see Table I in the Introduction to this Section.

H. LEVELS OF ANTITUBERCULOUS AGENTS IN BODY FLUIDS

On occasion, a patient whose organisms show *in vitro* susceptibility to drugs may fail to respond to treatment with these agents. There may be several explanations for such "treatment failures"; however, these will not be discussed here. Insufficient serum levels of the drug(s) may be one explanation for this phenomenon, and this is mentioned only because the bacteriologist may be asked to determine the level of certain anti-tuberculosis agents in various body fluids. If the clinician should request serum level determinations of antituberculosis drugs, relatively simple biological and chemical methods of drug assay are described by Stottmeier et al. (1967, 1968, 1969).

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SECTION IV. RECORDS AND REPORTS

The laboratory worker is responsible for keeping accurate records and reporting the actual results obtained by laboratory procedures. The worker does not interpret the results, since the physician has other information about the patient and is responsible for making the diagnosis. With careful observation and the test results, the laboratory worker should be able to make some distinction between the clinically significant and the clinically insignificant mycobacteria.

For laboratory results to be useful to the clinician in the diagnosis and treatment of the patient, uniform methods of reporting laboratory procedures are essential. Since laboratory tests in mycobacteriology are time-consuming, interim reports should be sent. A recommended reporting schedule follows:

First, report smear results as soon as they are available. This report should include: (1) the staining method used and (2) some indication of the number of acid-fast bacilli seen on the smear.

Later, at 3 to 4 weeks, report: (1) results of direct drug susceptibility tests, (2) amount of growth on the culture, and (3) a tentative or final identification of the species isolated.

Send the final report upon identification of the species. Cultures should be held 6 to 8 weeks before they are reported as negative.

For examples of reports, see Figures 22, 23, 24, 25, 26, and 27.

Figure 26
SAMPLE LABORATORY REPORT FORM

LABORATORY (Name)		FORM (Name)	
Please print with BLACK MEDIUM SOFT lead pencil only		Form No.	
Patient's Name JOHN DOE		Age 69	Sex M
Patient's Address SOMEWHERE U.S.A.		Race W	
Patient's Social Security Number or Occupation		County Code	
Physician Code		To be filled in by physician	
This is your return address		Mo. Day Year Date Specimen Collected	
DR J. J. JAY		SPECIMEN: Sputum: Natural: ☒ Nebulized: ☒	
TO Sanatorium		IS PATIENT ON THERAPY? ☒ Yes ☐ No	
Somewhere State, U.S.A.		WHAT DRUGS? ☒ INH ☐ VM ☒ SM ☐ ETA ☐ PAS ☐ CS ☐ KM ☐ ETH	
PLEASE PRINT CAREFULLY		If repeat, date last specimen submitted	
Laboratory Report		Mo. Day Year	
EXAMINED MICROSCOPICALLY BY: ☒ FIEHL NEELSEN ☐ FLUOROCHROME		First Repeat	
ACID-FAST BACILLI Found ☐ Not Found ☒ Rate ☒ Few ☐ Numerous		First Repeat	
FUNGUS OR YEAST Found ☐ Not Found ☐ Split Given To: Mycology Lab.		First Repeat	
FINAL REPORT MAY REQUIRE 8 WEEKS		First Repeat	
SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE ☐ Insufficient Amount ☐ Leaked in Transit ☐ Improper Container ☐ Contaminated		First Repeat	
DATE RECEIVED AND LABORATORY SPECIMEN NUMBER Month Day Year Form No. Specimen Number		DATE REPORTED 1st and 3rd Form Control Number	

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Figure 27
SAMPLE LABORATORY REPORT FORM

LABORATORY (Name)		FORM (Name)																																											
Please print with BLACK MEDIUM SOFT lead pencil only																																													
Patient's Name MARY DOE		Age 39	Sex F																																										
Patient's Address BIG GAP USA		Race W																																											
Patient's Social Security Number or Occupation		County Code																																											
Physician Code		To be filled in by physician																																											
This is your return address		Mo Day Year Date Specimen Collected																																											
DR. ☒		SPECIMEN: Sputum: ☐ Natural ☒ Nebulized ☐ Gastric ☐ Urine ☐ Spinal ☐ Pleural ☐ Culture																																											
State (Zip Code)		IS PATIENT ON THERAPY? Yes ☐ No ☐ WHAT DRUGS? ☒ INH ☐ VM ☐ SM ☐ ETA ☐ PAS ☐ CS ☐ KM ☐ ETH																																											
PLEASE PRINT CAREFULLY		If repeat, date last specimen submitted																																											
First Repeat		Mo Day Year																																											
Laboratory Report EXAMINED MICROSCOPICALLY BY: ZIEHL-NEESEN ☒ FLUOROCHROME ACID FAST BACILLI Found Not Found ☐ Rare ☒ Few ☐ Numerous FUNGUS OR YEAST Found Not Found Split Given To: Microbiology Lab		DRUG SUSCEPTIBILITY TESTS IN 7H-10 AGAR ☒ DIRECT ☐ INDIRECT																																											
FINAL REPORT MAY REQUIRE 8 WEEKS SPECIMEN UNSATISFACTORY FOR TESTING BECAUSE ☐ Insufficient Amount ☐ Leaked in Transit ☐ Improper Container ☐ Contaminated		<table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <thead> <tr> <th>Drug (µg/ml)</th> <th>Growth* at Dils</th> <th>Percent of Pop</th> </tr> <tr> <th></th> <th>10⁻¹</th> <th>10⁻³</th> </tr> </thead> <tbody> <tr> <td>CONTROL 0.0</td> <td>3+</td> <td>1+</td> </tr> <tr> <td>INH 0.2</td> <td>0</td> <td>0</td> </tr> <tr> <td>INH 1.0</td> <td></td> <td></td> </tr> <tr> <td>SM 2.0</td> <td>0</td> <td>0</td> </tr> <tr> <td>SM 10.0</td> <td></td> <td></td> </tr> <tr> <td>PAS 2.0</td> <td>0</td> <td>0</td> </tr> <tr> <td>PAS 10.0</td> <td></td> <td></td> </tr> <tr> <td>KM 5.0</td> <td></td> <td></td> </tr> <tr> <td>VM 5.0</td> <td></td> <td></td> </tr> <tr> <td>ETA 5.0</td> <td></td> <td></td> </tr> <tr> <td>CS 20.0</td> <td></td> <td></td> </tr> <tr> <td>ETH 2.0</td> <td></td> <td></td> </tr> </tbody> </table> *4+ = > 500 3+ = 200-500 TENTATIVE 2+ = 100-200 1+ = 50-100 IDENTIFICATION - 50 recorded & followed by "C" as 10C = 10 colonies		Drug (µg/ml)	Growth* at Dils	Percent of Pop		10 ⁻¹	10 ⁻³	CONTROL 0.0	3+	1+	INH 0.2	0	0	INH 1.0			SM 2.0	0	0	SM 10.0			PAS 2.0	0	0	PAS 10.0			KM 5.0			VM 5.0			ETA 5.0			CS 20.0			ETH 2.0		
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IDENTIFICATION ☒ M. tuberculosis ☐ M. bovis ☐ M. kansasii RUNYON GROUP II SCOTOCHROMOGEN ☐ M. macroflavescens ☐ Tap water RUNYON GROUP III NON-PHOTOCHROMOGEN ☐ M. intracellulare ☐ M. terrae ☐ M. goodii ☐ M. triviale RUNYON GROUP IV RAPID GROWERS ☐ M. fortuitum ☐ M. flavescens ☐ Other		Date Received and Laboratory Specimen Number Month Day Year Form No. Specimen Number																																											
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**APPENDIX A:
POSTAL REGULATIONS**

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APPENDIX A:

POSTAL REGULATIONS

Postal Requirements for Shipping Diseased Tissues and Other Specimens

(Extracts from Parts 124, 125, and 221, United States Postal Manual)

PART 124

NONMAILABLE MATTER

124.1 INTRODUCTION

.11 DESCRIPTION. Nonmailable matter includes all matter which is by law, regulation, or treaty stipulation prohibited from being sent in the mail or which cannot be forwarded to its destination because of illegible, incorrect, or insufficient address.

.12 APPLICABILITY. The harmful or objectionable things identified or described in this part are some of the matter which may not be sent through the mail, as a matter of absolute prohibition. See part 125 for matter mailable only under special rules or conditions. Notwithstanding any statement contained in part 124, which covers only some of the items prohibited in the mail, the burden rests with the mailer to assure that he has complied with the law. In addition to the nonmailable items mentioned in this part, certain other articles are prohibited in the mail to military post offices overseas (part 127).

.13 PENALTIES FOR VIOLATION. Severe penalties, by fine or imprisonment, or both, are provided for persons who knowingly mail or cause to be mailed, any matter which has been declared nonmailable under law.

.14 NONCONFORMITY WITH POSTAL REGULATIONS. Regardless of its nature, matter may not be mailed in any form if done in violation of postal regulations for such reasons as failure to pay postage, improper size or weight, improper permits, improper addresses, etc.

.15 RESPONSIBILITY OF MAILER. When mailers are in doubt as to whether any matter is properly mailable, they should ask the postmaster. Even though the Post Office Department has not expressly declared any matter to be nonmailable, the mailer of such matter may be held fully liable for violation of law if he does actually send nonmailable matter through the mail.

124.2 HARMFUL MATTER

.21 GENERAL PROVISIONS OF LAW

Any articles, compositions, or materials, which may kill or injure

another, or injure the mail or other property, are nonmailable. This includes but is not limited to:

- a. All kinds of poison or matter containing poison.
- b. All poisonous animals, except scorpions (see 125.35), all poisonous insects, all poisonous reptiles, and all kinds of snakes.
- c. All disease germs or scabs.
- d. All explosives, flammable material, infernal machines, and mechanical, chemical, or other devices or compositions which may ignite or explode.

* * *

PART 125

MATTER MAILABLE UNDER SPECIAL RULES

125.1 LEGAL RESTRICTIONS

.11 HARMFUL MATTER

.111 Certain items barred from the mail, as set forth in part 124, may be mailed if prepared and packaged in accordance with this part. These are items not outwardly or of their own force dangerous or injurious to life, health, or property.

.112 This part covers generally some of the more common situations; however, the burden rests with the mailer to assure that he has complied with the law that anything shipped by him has been properly prepared and packaged. The ordinary test of adequate preparation and packaging is whether the contents of a parcel are safely preserved under ordinary hazards of mail handling and transportation.

.113 Products, materials, and devices are created or modified with such frequency that the Post Office Department is unable to issue general rulings in advance to govern adequate preparation and packaging. Any mailer may, however, request the Post Office Department, in advance, for a specific ruling as to mailability of his item. The request should be addressed to the local postmaster, who will forward it to the Classification and Special Services Division, Bureau of Operations, Washington, D.C. 20260.

.12 APPLICABILITY OF OTHER LAWS

* * *

.122 Any special conditions or limitations placed on transportation or movement of certain things shall govern admissibility to the United States mail, when imposed under law by the U. S. Department of the Treasury; U. S. Department of Agriculture; U. S. Department of Commerce; U. S. Department of Health, Education, and Welfare; Interstate Commerce Commission; or any other Federal department or agency having legal jurisdiction.

.13 PENALTIES

Severe penalties of fine or imprisonment, or both, are provided by law, for anyone who knowingly deposits for mailing or delivery, or causes to be mailed or delivered, anything declared nonmailable under law. Failure to comply with the regulations of the Postmaster General, as prescribed in this part, as to matter otherwise nonmailable, constitutes a violation of law.

125.2 CONDITIONS FOR MAILING

.21 GENERAL NATURE OF PRECAUTIONS REQUIRED

.211 The restrictions against mailing of harmful matter, from which relief is granted by this part, are intended to prevent damage or harm to postal and transportation personnel, to prevent damage or destruction of other mail and of property, to avoid obnoxious odors, and to prevent the spread of disease and infection. Special preparation and packaging are required to protect against such contingencies.

.212 Basic precautions, covered generally in this section, relate to the inner containers holding the harmful matter, internal cushioning and protection, and exterior packaging and marking.

.22 LIQUIDS (NONFLAMMABLE) AND POWDERS

.221 Precautions shall be taken in the case of liquids, pastes, salves, ink powders, pepper, snuff, or other pulverized materials against damage to mail and property from leakage and against caustic, irritant, toxic, or soiling effect on mail handling personnel.

.222 Container shall meet any applicable Interstate Commerce Commission or other Federal specifications. Closures must effectively seal the contents against leakage. Friction tops must be fastened with solder, clips, or otherwise so that they will not come off under impact.

.223 Glass or other breakable containers of liquid must be packaged to withstand handling enroute. The container shall be cushioned inside the carton to absorb shock and impact. Where feasible, absorbent material shall be used, to take up all the liquid in case of breakage.

* * *

125.7 IDENTIFICATION AND MARKING

.71 IDENTIFICATION OF CONTENTS. The identity or nature of contents of anything mailed under any of the provisions of part 125 shall be stated plainly on the outside of the parcel, as a condition of mailing.

.72 IDENTIFICATION OF MAILER AND ADDRESSEE. The full name and address of both the mailer and the addressee shall be written in ink, rubberstamped, or pasted on the outside of any package whose mailing is covered by part 125.

.73 LABELS. Any labels required under Federal law or under any regulations issued by any Federal agencies pursuant to Federal law shall be pasted to the outside of the parcel.

* * *

PART 221

CONDITIONS APPLICABLE TO ALL CLASSES

221.1 PREPARING AND ADDRESSING

.11 PREPARING

.111 Senders must prepare articles securely, especially if they are for distant countries. International mail is handled more often and subjected to greater pressure and friction than domestic mail, hence it must be enclosed in strong envelopes or other wrappings.

* * *

.114 Articles other than letters and letter packages (AO mail) must be prepared in such a way that their contents are sufficiently protected but so as not to hinder quick and easy inspection of the contents. They should be placed under wrapper, on a roller, or between cardboard; in open bags, boxes, envelopes, or containers or in closed, unsealed bags, boxes, envelopes, or containers provided with fasteners that can be easily opened and reclosed without being dangerous; or they may be tied with string or twine in a manner that will permit them to be easily untied. Sealing of postal union *other articles* is not permitted, even if registered, and they must be prepared in such a way that other articles do not run the risk of being trapped by them.

* * *

221.3 PROHIBITIONS AND RESTRICTIONS

.32 RESTRICTED ARTICLES

* * *

.325 *Perishable Biological Materials*. Perishable biological materials, including those of pathogenic nature, when sent in the postal union mail are accepted only as LETTER PACKAGES. The following conditions apply:

a. *Mailing Restrictions*

If a country prohibits perishable biological materials this is shown under *Prohibitions* in the country item in the Directory of International Mail. The packages must be packed as prescribed in 221.325c and must bear distinctive violet labels by which they can be readily recognized and receive careful handling and prompt delivery.

b. *Qualification of Mailers*

(1) Only officially recognized laboratories may send or receive letter packages containing perishable biological materials. Laboratories of the following categories are so designated:

Laboratories of local, State, and Federal government agencies.

Laboratories of federally licensed manufacturers of biologic substances derived from bacteria and viruses. Laboratories affiliated with or operated by hospitals, universities, research facilities, and other teaching institutions.

Private laboratories licensed, certified, recognized, or approved by a public authority.

- (2) A laboratory desiring to mail letter packages containing materials of this kind shall make written application on its letterhead stationery to the Classification and Special Services Division, Bureau of Operations, Post Office Department, Washington, D.C. 20260, explaining its qualifications and those of the prospective addressee to send and receive such materials, and stating how many packages are to be mailed. On approval, the mailer will receive a sufficient number of the violet labels for the contemplated shipments.

c. Packaging

- (1) Perishable biological material not of a pathogenic nature must be packed in a nonporous container surrounded by sufficient absorbent material to take up all the liquid and must be placed in an outer protective container where it should fit tightly to avoid any shifting.
- (2) Perishable biological material of a pathogenic nature must be packed in a tightly closed bottle or tube or heavy glass wrapped in thick absorbent material rolled several times around the bottle or tube and tied at the ends, sufficient in quantity to absorb all the liquid; the wrapped container must be placed in a strong well-closed metal box constructed to prevent any contamination outside of it. This metal box must be wrapped in cushioning material and placed in an outer protective box where it should fit tightly to avoid shifting. The outer container must consist of a hollow block of strong wood, metal, or other equally strong material with a tight lid so fitted that it cannot open during transportation.
- (3) In addition to the requirements in (1) and (2), packages must comply with the regulations governing the transmission of such materials in the domestic mail.
- (4) The mailer must place on each package one of the violet labels mentioned in a and b(2).

* * *

**APPENDIX B:
GENERAL EQUIPMENT**

APPENDIX B:

GENERAL EQUIPMENT

The items of equipment, glassware, chemicals, and other materials listed are generally found in most laboratories. Any specifications given are those required in the methods and procedures described in this manual.

EQUIPMENT

Analytical balances
Autoclave, steam or electric
Beads, glass or plastic
Burner, Bunsen or Fisher
Carbon dioxide gauge, e.g., Fyrite or Burwell
Centrifuge with tachometer, 3000 rpm (1800-2400 *g*)
Desiccator, vacuum
Discard cans, splash proof
Discard pans, with covers
Filter pump
Filters, bacteriological (Seitz and Millipore)
Freezer: -20°C to -40°C
Hand lens
Incubators, fitted for carbon dioxide
Inoculating spade or loop
Inspissator (for egg medium if used)
Interval timers
Microscope, dissecting, 10-50X for use with transmitted light
Microscope for fluorescence microscopy
Microscope with oil immersion lens
Mixer, test tube, high speed, e.g., Vortex or other
pH meter
Pipetting unit, Cornwall
Racks for specimen tubes
Refrigerator, 4 to 10°C
Safety equipment (See section on Safety.)
Slide staining apparatus
Slide warming table, Ranson or equivalent
Temperature block heaters 37°C and 68°C
Thermometers
Turntable for inoculating plates
Water baths 37°C and 56°C

GLASSWARE

Bottles for stains

Cylinders, graduated, 25 ml to 1000 ml

Flasks, Erlenmeyer or other, 50 ml to 3000 ml

Flasks, Volumetric, 100 ml, 500 ml, 1000 ml

Pipettes

Capillary (Pasteur type)

1 ml graduated in 1/100 ml

5 ml graduated in 1/10 ml

10 ml graduated in 1/10 ml

Slides, microscope

27 x 75 mm frosted end, regular

27 x 75 mm for fluorescence microscopy

Tubes, screw-cap

16 x 125 mm

18 x 150 mm

50 ml centrifuge, aerosol free

PLASTICS

Bags, Mylar

Polyethylene

Centrifuge tubes, screw-cap, 50 ml, water-tight

Petri dishes

Plain 15 x 100 mm

1 plate (2 sections) 15 x 100 mm

X plate (4 sections) 15 x 100 mm

APPENDIX C:
CHEMICALS AND MEDIA BASES

APPENDIX C: CHEMICALS AND MEDIA BASES

Only chemicals of the highest purity should be used. A few may be available from only one manufacturer.

CHEMICALS

Agar
Albumin, Bovine Fraction V
Alcohol, 95% ethyl
Ammonium hydroxide
Ammonium sulfate
Aniline
Asparagine
Auramine O
Barium chloride
Biotin
Brom thymol blue (dibrom thymol sulfon phthalein)
Calcium chloride ($2\text{H}_2\text{O}$)
Catalase, technical grade
Copper sulfate ($5\text{H}_2\text{O}$)
Cyanogen bromide
Dextrose (glucose)
Disodium phosphate (anhydrous)
Ferric ammonium citrate
Fuchsin, basic, 94% dye content
Glycerol, reagent grade
Hydrochloric acid
Hydrogen peroxide (30% Superoxol)
Magnesium citrate
Magnesium sulfate ($7\text{H}_2\text{O}$)
Malachite green oxalate
Methylene blue chloride
Monopotassium phosphate (anhydrous)
Monosodium glutamate
N-acetyl-L-cysteine powder
Neutral red
N-naphthylethylenediamine dihydrochloride
Oleic acid
Phenol crystals, reagent grade
Phenol liquid
Phenolphthalein
Phenolsulfonphthalein, sodium salt (phenol red)
Potassium permanganate
Potassium tellurite
Potato flour or potato starch

Pyridoxine hydrochloride
Rhodamine B
Sodium carbonate
Sodium chloride
Sodium citrate
Sodium hydroxide
Sodium nitrate
Sulfanilamide
Sulfuric acid
Tripotassium phenolphthalein disulfate
Trisodium phosphate
Tween 80®
Zinc sulfate (7H₂O)

MEDIA BASES

Dubos broth base with ADC enrichment
MacConkey agar
Middlebrook 7H-9 broth base with ADC enrichment
Middlebrook 7H-10 agar base with OADC enrichment
Wayne sulfatase agar

**APPENDIX D:
COLOR STANDARDS**

APPENDIX D:
COLOR STANDARDS

Arylsulfatase Test Result Standards

M/15 Disodium phosphate, See p. 111.

0.1% stock phenol red: 0.1 gm phenolsulfonphthalein sodium salt in 100 ml distilled water. Autoclave for storage in the refrigerator.

Prepare six screw-cap test tubes (16 x 125) as follows:

Tube No.	M/15 Na_2HPO_4	0.1% Phenol Red	Reading
1	4 ml	10 drops 0.1% stock.	5+
2	4 ml	1 ml from tube 1	4+
3	3 ml	2 ml from tube 2	3+
4	3 ml	2 ml from tube 3	2+
5	3 ml	2 ml from tube 4	1+
6	3 ml	2 ml from tube 5	±

Discard 2 ml from tube 6, autoclave, seal, and store in the refrigerator. Color standard usually retains its color for 6 months.

Nitrate Reduction Standards

STOCK SOLUTIONS

1. M/15 disodium phosphate
2. M/15 monopotassium phosphate
3. M/15 trisodium phosphate
4. 1% phenolphthalein (1 gram in 100 ml 95% ethyl alcohol)
5. 1% brom thymol blue (1 gram in 100 ml 95% ethyl alcohol)
- 5A. 0.01% brom thymol blue: prepared by mixing 1.0 ml of stock No. 5 above in 100 ml distilled water.

WORKING BUFFER SOLUTION

Mix:

Stock No. 1	35 ml
Stock No. 2	5 ml
Stock No. 3	100 ml

PREPARATION OF STANDARDS

1. Line up eight clean test tubes in a rack.
Use tubes the same size as for the test.
2. Put 2 ml of working buffer solution into seven tubes.
3. To 10 ml of working buffer solution, add 0.1 ml of stock No. 4 and 0.2 ml of stock No. 5A.
4. Add 2 ml of the step 3 solution to the 8th tube. This is the 5+ color standard.
5. To the first tube in the series of seven tubes, add 2 ml of the step 3 solution. Mix well and carry over 2 ml to the next tube. Continue to make serial dilutions discarding 2 ml from the 7th tube.
6. The color standard:
tube in step 4 = 5+
tube 1 = 4+
tube 2 = 3+
tube 4 = 2+
tube 5 = 1+
tube 7 = ±
These colors should range from pink (±) to purplish red (5+).
7. Autoclave tubes, seal, and store in a refrigerator.

APPENDIX E.
PREPARATION OF REAGENTS AND MEDIA

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APPENDIX E:

PREPARATION OF REAGENTS AND MEDIA

1. M/15 Phosphate Buffer Solutions

Buffer solutions are prepared by mixing two stock solutions in proportions to give the required pH.

a. STOCK SOLUTIONS

- (1) M/15 disodium phosphate: In a volumetric flask dissolve 9.47 g of anhydrous Na_2HPO_4 in distilled water to make one liter (1000 ml).
- (2) M/15 monopotassium phosphate: In a volumetric flask dissolve 9.07 g of KH_2PO_4 in distilled water to make one liter (1000 ml).

b. pH 6.8 BUFFER SOLUTION

Mix 50.0 ml of (1) with 50 ml of (2).
Check on pH meter.

c. pH 7.0 BUFFER SOLUTION

Mix 61.1 ml of (1) with 38.9 ml of (2).
Check on pH meter.

Autoclave to sterilize.

2. M/15 Trisodium Phosphate

Make 25.33 g of $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ to 1 liter with distilled water.

3. 4% Sodium Hydroxide (1N)

Dissolve 40 g of NaOH in one liter (1000 ml) of distilled water. Autoclave and store at room temperature. If a precipitate forms, discard and prepare fresh solution.

4. 2.9% Sodium Citrate (0.1M)

Dissolve 29.4 g of anhydrous Na citrate in one liter of distilled water. Autoclave and store at room temperature. If a precipitate forms, discard and prepare fresh solution.

5. Physiological Saline (0.85%)

Dissolve 8.5 g sodium chloride in 1000 ml distilled water. Autoclave and store at room temperature.

6. 0.2% Bovine Albumin Fraction V

- (a) Dissolve 0.85 g sodium chloride in 100 ml of distilled water.
- (b) Add 2.0 g of bovine albumin fraction V (2% solution). Dissolve by swirling flask or by gently stirring on a magnetic stirrer.

- (c) Adjust pH to 6.8 with 10% NaOH.
 - (d) Sterilize by filtration.
 - (e) For 0.2% albumin solution dilute this sterile 2% solution 1:10 with sterile M/15 phosphate buffer pH 6.8. It is convenient to sterilize the phosphate buffer in 9 ml amounts in screw-cap tubes or bottles. Add 1 ml of the sterile albumin to the sterile buffer for a 0.2% solution.
- The use of small volumes of 0.2% albumin lessens the possibility of contaminating the albumin and, consequently, the specimens.

7. General Rules for Preparation and Storage of Media

For media of the best quality, use chemicals of certified purity, clean glassware, freshly distilled water, and follow the directions precisely.

To avoid contaminating reagents and media:

- (1) Keep the environment as clean as possible.
- (2) Use an isolated area and minimize traffic in the preparation room.
- (3) Swab the work surface with a disinfectant before dispensing sterile reagents and media.
- (4) Clean the floor with a wet mop. This will keep dust down.
- (5) If necessary, place ultrafilters over air intakes to reduce air contaminants.
- (6) Use high-intensity ultraviolet light to sterilize air when room is *not in use*. However, do *not* leave media exposed to *any* light rays.
- (7) Use sterile glassware and equipment.
- (8) Observe strict aseptic technique. Flame flasks and tubes when adding enrichment or drugs to the media.
- (9) Do not leave covers off Petri dishes after pouring the media into plates.
- (10) Be sure the Oleic acid Albumin Dextrose Complex (OADC enrichment) and drugs are properly sterilized before adding them to the sterile basal medium.
- (11) Check pore size of sterilizing filters to make certain that they are adequate.
- (12) Check temperature and pressure of autoclave.
- (13) When preparing eggs to add to egg-base media, be sure that the shells of the eggs are carefully cleaned before breaking.

8. Storage of Media

- (1) Always shield any media from direct daylight.

- (2) Place plates medium-side *down* in clean plastic bags or sterile dry cannisters.
- (3) Before storage, tighten tops of screw-cap tubes.
- (4) Media to be used within 1 week after preparation may be stored at room temperature *in the dark*.
- (5) To prevent dehydration, any media more than 1 week old should be stored in the refrigerator.
- (6) Properly stored media may be kept 2 to 3 months.
- (7) Discard any unused media that shows signs of dehydration or contamination.

9. Preparation of 7H-10 Medium from Basic Ingredients

Six stock solutions may be prepared in advance. When prepared and stored as indicated, these solutions remain stable for at least a month. If precipitation or flocculation occurs in any stock solution, discard and prepare fresh solutions.

a. TO PREPARE THE SIX SOLUTIONS

Solution No. 1

Monopotassium phosphate, A.C.S. (anhydrous) 15.0 g
 Disodium phosphate, A.C.S. (anhydrous) 15.0 g
 Distilled water to make 250.0 ml
 Autoclave at 121°C for 15 minutes.

Store this solution at room temperature.

Solution No. 2

Ammonium sulfate, A.C.S. 5.0 g
 Monosodium glutamate
 (glutamic acid, monosodium salt) 5.0 g
 Sodium citrate (2H₂O) USP 4.0 g
 Ferric ammonium citrate (brown scales) 0.4 g
 Magnesium sulfate (7H₂O), A.C.S. 0.5 g
 Biotin (in 2 ml of 10% ammonium hydroxide) 5.0 mg
 Distilled water to make 250.0 ml

Autoclave at 121°C for 15 minutes.

Store in the refrigerator at 4-10°C.

Solution No. 3

Calcium chloride (2H₂O), A.C.S. 50 mg
 Zinc sulfate (7H₂O), A.C.S. 100 mg
 Copper sulfate (5H₂O), A.C.S. 100 mg
 Pyridoxine hydrochloride 100 mg
 Distilled water to make 100 ml

Store in the refrigerator at 4-10°C.

Solution No. 4

Reagent grade glycerol. Store at room temperature.

Solution No. 5

Aqueous malachite green—0.01%

Dissolve 0.1 g malachite green in 100 ml distilled water.

Dilute 1:10 to make 0.01%.

Store at room temperature or 4-10°C *in the dark*.

Solution No. 6

Oleate-Albumin-Dextrose-Catalase (OADC)

(1) Dissolve 50 g bovine albumin fraction V in 900 ml freshly prepared saline (0.85% NaCl).

(2) Add 30 ml sodium oleate prepared as follows:

N 20 NaOH 30.0 ml

Oleic acid 0.6 ml

Warm to 56°C and swirl gently to dissolve.

(3) Adjust pH to 7.0 with 4% NaOH.

(4) Heat in a water bath at 56°C for 1 hour.

(5) Add 40 ml of sterile 50% solution of dextrose prepared as follows:

To 30 ml of boiling distilled water, add 25 g of dextrose (glucose). Stir to dissolve.

Add distilled water to make 50 ml total volume.

Autoclave 10 minutes at 121°C.

(6) Add 0.02 ml technical catalase.*

(7) Sterilize by membrane filter. To facilitate this process, use a pre-filter with the sterilizing membrane (0.45 micron) and filter while the albumin is still warm.

(8) Dispense in sterile screw-cap tubes in 20 ml or other convenient amounts.

(9) Incubate 24 hours at 35-37°C. If sterile, the mixture is ready for use.

(10) Store in the refrigerator in air-tight containers. Do not freeze.

Note: *If it is inconvenient to prepare solution No. 6, the commercially available OADC is an acceptable substitute.*

* Catalase is inactivated by Seitz and sintered glass filters. If membrane filter sterilization is unavailable, omit the catalase from solution No. 6 and add it to the sterile completed medium.

b. TO PREPARE 800 ml COMPLETED MEDIA FROM THE SIX STOCK SOLUTIONS (OR FOUR "BATCHES" OF 200 ml EACH):

- (1) Distilled water 680.0 ml
- Solution No. 1 20.0 ml
- Solution No. 2 20.0 ml
- Solution No. 3 0.8 ml
- Solution No. 4 4.0 ml
- (2) Adjust the pH to 6.6 by adding approximately 0.4 ml of 6N HCl. Check on pH meter.
- (3) Add solution No. 5. 2.0 ml
- (4) Divide into four equal parts of 180 ml each.
- (5) To each 180 ml, add agar 3.0 g
- (6) Autoclave at 121°C for 10 minutes.
- (7) Cool to 56°C (for drug media, cool to 50-52°C).
- (8) Add to each 180 ml flask:
 - (a) Solution No. 6 20.0 ml
 - (b) If catalase is not in OADC, prepare a sterile solution containing 1000 mcg/ml and add 0.4 ml*
 - (c) For drug media, add sterile drug.
- (9) Mix gently but thoroughly and dispense into Petri dishes. (20 ml per dish or 5 to 6 ml per quadrant in four-section dish). *Media must be cooled and dispensed within 1 hour after autoclaving.*
- (10) Allow to solidify at room temperature *in the dark*.

* To prepare sterile catalase, add 0.02 ml catalase to 20 ml 0.85% saline; this solution contains 1000 mcg/ml. Sterilize by membrane filter. To each 100 ml of completed medium, add 0.2 ml of the sterile catalase solution.

10. Preparation of 7H-10 Agar from Commercial Base

Check the bottle label for this reference, "Acta Tuberc. Scand. 38:66, 1960." This reference on the label indicates that the bottle contains the correct ingredients.

Note: *Experience has shown that boiling the basal medium before autoclaving results in a medium of inferior quality. This result may be avoided by preparing the medium in 200 ml amounts and autoclaving for 10 minutes.*

To prepare 200 ml completed medium follow these directions precisely:

- (1) Suspend 3.8 g* of Middlebrook 7H-10 basal medium in 180 ml of freshly distilled water and add 1.0 ml of reagent grade glycerol.
- (2) Swirl base into suspension and sterilize in the autoclave for 10 minutes at 121°C.
- (3) Remove medium from autoclave *as soon as* the pressure will allow and place in a water bath at 50-56°C.
- (4) As soon as cooled to 50-56°C, add 20 ml of OADC enrichment.
- (5) For drug media, cool to 50-52°C, then add the required amount of drug.
- (6) Dispense *within 1 hour after autoclaving*.
- (7) Allow to solidify at room temperature *in the dark*.

Note: When the autoclaved medium is allowed to stand for more than 1 hour in the water bath, a precipitate begins to form. Experience has shown that this practice should be avoided. Pour as soon as possible. Always check the medium when taking it out of the autoclave. It should be clear and transparent. If a precipitate is present, it may be due to: (a) sterilization temperature within the autoclave being reached too rapidly to dissolve the base in 10 minutes, (b) poor quality basal medium and/or (c) dirty glassware.

Caution: Never autoclave 7H-10 medium base and store it in the refrigerator for future use. The heat required to melt it results in overheating and a medium of poor quality.

For best results do not prepare in lots of more than 400 ml.

* Calculated from directions on the label for formula in Acta. Tuberc. Scand. 38:66, 1960.

11. Preparation of Tween-Albumin Liquid Medium

The Middlebrook 7H-9 and Dubos-Middlebrook Tween-albumin liquid media are satisfactory as supplied by commercial sources.

Prepare according to directions on the label. Tube in 5 ml quantities in 18 x 150 mm sterile screw-cap test tubes. Incubate for sterility check.

Liquid media may be stored in the refrigerator indefinitely, provided it is tightly capped and evaporation does not occur.

12. Preparation of Modified Lowenstein Egg Medium

- (1) For salt solution combine:

Monopotassium phosphate (anhydrous)	2.4 g
Magnesium sulfate (7H ₂ O)	0.24 g
Magnesium citrate	0.6 g
Asparagine	3.6 g
Glycerol (reagent grade)	12.0 ml
Distilled water	600.0 ml

- (2) Add potato flour 30.0 g

- (3) Autoclave at 121°C for 30 minutes.

Cool to room temperature.

- (4) Add malachite green (2% aqueous solution freshly prepared) 20.0 ml

- (5) Add homogenized whole eggs 1000.0 ml*

- (6) Mix and pour into a sterile aspirator bottle or funnel with a bell attachment (test tube filling device) and dispense.

- (7) Place approximately 6-8 ml medium into each 18 x 150 mm sterile screw-cap test tube.

- (8) Slant tubes and coagulate by inspissation at 85°C for 50 minutes.

- (9) Incubate at 37°C for 48 hours as a sterility check.

- (10) Medium may be stored in the refrigerator for several months if caps are tightly closed to prevent evaporation.

* Fresh eggs, not more than 1 week old, are cleaned by scrubbing with a hand brush in a soap solution (about 5%). After scrubbing, the eggs are allowed to stand in the soap solution for 30 minutes. Rinse thoroughly in running water, then soak eggs in 70% alcohol for 15 minutes. With well scrubbed hands, break the eggs into a sterile flask and homogenize by hand shaking. Filter eggs through 4 layers of sterile gauze into a sterile graduated cylinder.

13. Preparation of American Trudeau Society (ATS) Medium*

a. INGREDIENTS

Potato flour	20.0 g
Distilled water	490.0 g
Glycerol	10.0 ml
Egg yolk	500.0 ml
1% malachite green in 50% alcohol	20.0 ml

b. PROCEDURE

- (1) Combine potato flour, water, and glycerol in a 2-liter flask.
- (2) Autoclave at 121°C for 30 minutes.
- (3) Cool to 50°C.
- (4) *MEANWHILE*: Prepare the fresh egg yolk as follows: Carefully clean fresh hen eggs as described in a footnote to the section on preparing Lowenstein medium, page Using aseptic techniques, separate the egg yolk from the white. For the 500 ml egg yolk, use a proportion of one whole egg to 11 yolks.
- (5) Combine the homogenized egg yolk mixture with the cooled potato flour water.
- (6) Add 20.0 ml malachite green. Mix thoroughly.
- (7) Dispense 5-6 ml per tube into sterile screw-cap tubes.
- (8) Slant and coagulate by inspissation at 85°C for 1 hour.

* For the sodium chloride tolerance test, add 50.0 g of sodium chloride to the potato flour, water, and glycerol before heating in Step 2. This gives a final concentration of 5% in the completed medium.